PHYSIOLOGICAL EFFECTS OF THE PARASITE *ICHTHYOPHONUS* ON SPAWNING CHINOOK SALMON AND THEIR OFFSPRING IN A YUKON RIVER TRIBUTARY

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7 December 2015
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A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

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Fairbanks, AK

December 2015

Abstract

In recent years, Chinook salmon *Oncorhynchus tshawytscha* returns to the Yukon River, Alaska, have been substantially reduced. In summer 2010 – 2012, spawning Chinook salmon (*n*=51, 32, and 23, respectively) were collected from the Salcha River, a tributary of the Yukon River, to determine the effects of *Ichthyophonus,* a protozoan parasite, on salmon reproductive success. Eggs and milt from *Ichthyophonus*-infected and non-infected parents were collected in 2010 and cross-fertilized to investigate offspring survival and potential second-generation effects induced by the parasite. Proximate composition analysis of adult muscle, eggs, and alevins, and blood chemistry analysis of adult blood plasma and alevin whole body homogenates were analyzed to explore potential differences between *Ichthyophonus*-infected and non-infected salmon. *Ichthyophonus* infection prevalence was 7.8, 6.3, and 8.3 % in 2010, 2011, and 2012, respectively. Egg lipid content was significantly higher in eggs from *Ichthyophonus*-infected females, compared to eggs from *Ichthyophonus*-negative females. Survival of fertilized eggs to hatching was not significantly different between offspring from *Ichthyophonus*-infected parents (Mean±1SD: 24.4±29.8 % survival) and non-infected parents (41.0±24.8 % survival). Proximate composition (% lipid, % protein, kJ/g) of muscle from spawning adult salmon also did not differ, nor did total body composition or morphology of alevins produced by either *Ichthyophonus*-infected or non-infected parents. We found no significant differences in blood plasma cortisol concentrations (a stress indicator) between *Ichthyophonus*-positive and negative adults or their offspring. There were also no significant differences in blood chemistry parameters indicative of tissue damage between *Ichthyophonus*-positive and *Ichthyophonus*-negative adults or resulting alevins, with the exception of aspartate aminotransferase, which was unexpectedly higher in plasma of *Ichthyophonus*-negative adults. Overall, infection with *Ichthyophonus* does not appear to impact the spawning ability or spawning success of Chinook salmon in the Salcha River.

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Acknowledgements

I would like to acknowledge the following people, agencies, and institutions for their support and contributions to this thesis:

My graduate advisor, Dr. Lara Horstmann and my graduate committee, Dr. Shannon Atkinson, Dr. Trent Sutton, and Mr. Calvin Skaugstad for their support in seeing me through my graduate career, including the many reviews of this thesis.

The Alaska Department of Fish and Game, particularly Ms. April Behr, Mr. Travis Hyer, and Mr. Thomas Redington for their help with obtaining samples for this project.

Dr. Arny Blanchard, Ms. Jill Seymour, Mr. Brian Walker, Ms. Stacy Vega, Ms. Nicole Farnham, Ms. Kendall Mashburn, Ms. Patty McCall, Mr. Kevin Fraley, and Mrs. Angie Steeves for support with sample collection and analyses.

The Pollock Conservation Cooperative Research Center for financial support.

Dedication

There are a number of people without whom this thesis might not have been written, and to whom I am greatly thankful. This volume is dedicated to those individuals whose support and wisdom allowed me to realize this success.

I am forever indebted to my mentors, Dr. Lara Horstmann who gave me the opportunity to pursue this adventure and took a chance on a Florida girl, and Dr. Shannon Atkinson for encouragement and moral support throughout the years.

I am eternally grateful to my parents, Ruth and Sidney, who have been a constant source of confidence and inspiration to me throughout my life, a very special thank-you for nurturing me through the months of writing.

To my sister, Maxine, who always commiserated and empathized with me during my graduate career, your shoulder should be lighter and dryer in months to come.

To my husband, Sean, who was there for me every step of the way. This has been a difficult but adventurous journey. Your boundless patience, support, encouragement, and enduring love have kept me swimming, when I thought I would sink. Not many people would move more than 5000 miles away with a girl they met a few months before to go to America's last frontier, where running water is a luxury. I love that you endured -40+ winters with me and helped me with so many things, even the outhouse monster! I love you more than words can say.

Finally to all my other family and friends who have given support and encouragement throughout my graduate career, you guys are the best.

Chapter 1: General Introduction

Chinook salmon *Oncorhynchus tshawytscha* are the largest of the eight Pacific salmon species and can reach nearly 45 kg (Healey 1991, Quinn 2005). They are distinguished by small black spots on both sides of their caudal fin, and black coloration along their gums (Healey 1991). Chinook salmon are unique from other salmon species due to their varying flesh color, which can be close to white to varying shades of pink, and even red (Healey 1991). Uptake and deposition of carotenoids in muscle of Chinook salmon is a heritable quantitative trait that is responsible for the color of the flesh (Gjerde and Schaeffer 1989, Quinton et al. 2005).

Chinook salmon have two behavioral populations, 'stream-type' and 'ocean-type' (Gilbert 1922, Quinn 2005). Stream-type Chinook salmon are mostly observed in Asian populations, northern populations in North America, and the headwater tributaries of southern populations in North America (Healey 1991). These fish spend at least one year as juveniles or fry in freshwater *prior* to their migration to sea. Stream-type Chinook salmon adults return to the freshwater several months before spawning, with runs occurring between February and July (Healey 1991). In contrast, ocean-type Chinook salmon populations are usually found on the North American coast south of 56 °N and migrate to sea in their first year of life, usually during the first 90 days after hatching (Healey 1991, Quinn 2005). Ocean-type Chinook salmon adults return to their natal streams and spawn soon after entering freshwater (Healey 1991). Their runs generally occur later in the year, beginning in July and extending to the fall, and these fish generally have a lower fecundity than the stream-type Chinook salmon (Healey 1991). Chinook salmon in the Yukon River drainage follow the stream-type life history behavior (Healey 1991); once hatched, most fish migrate to the ocean about one year after hatching, with some delaying migration for an additional year (Behnke 2002). These two behavioral forms reflect different adaptations to uncertainties in Chinook salmon juvenile survival and productivity. Stream-type juvenile Chinook salmon spend more time in relatively safer, but less nutrient-rich freshwater of their natal spawning stream to grow larger before migrating to sea (Healey 1991, Clarke et al. 1994). On the other hand, juvenile ocean-type Chinook salmon migrate earlier to food-rich estuarine habitats, where they are at larger predation risk, but may grow more rapidly (Healey 1991, Clarke et al. 1994).

Chinook salmon in Alaska are primarily stream-type (Behnke 2002). Before spawning, female Chinook salmon excavate a superficial dimple in the stream bed gravel by carrying out forceful swimming actions on the side close to the bottom substrate (Healey 1991). When the female spawns, she deposits a group or 'pocket' of eggs into the dimple and conceals them with gravel. Over the next several days, the female will add up to five more egg pockets in a row going upstream, making the dimples larger as she heads further upstream (Healey 1991). The entire area of diggings are termed the 'redd' (Healey 1991, Quinn 2005), and eggs are fertilized immediately after deposition. Alevins emerge from the gravel beds in late winter or early spring depending on time of fertilization and water temperatures (Healey 1991). Juvenile Chinook salmon overwinter in their natal freshwater habitat and then begin their migration to sea during the spring and into the summer the year after their emergence as alevins (Healey 1991, Burke et al. 2013). Chinook salmon juveniles generally reside in estuarine areas to rear until they reach smolt size or about 70 mm fork length. After this size, they move on to nearby marine areas (Healey 1991). The range of Alaska Chinook salmon spans the entire Bering Sea during maturation (Healey 1991), where they may stay in the marine environment for two to six years before returning to freshwater to spawn (Healey 1991).

The Yukon River begins at the Llewellyn Glacier, close to Atlin Lake, located in northwestern British Columbia, Canada, about 50 km from the Gulf of Alaska. From the Llewellyn Glacier, the Yukon River runs northwest through the Yukon Territory and British Columbia, Canada, traversing west through Alaska ending at the Bering Sea. The Yukon River serves as a transportation highway across the vast and roadless northern parts of Alaska (Brabets et al. 2000). Salmon species migrate throughout the over 3200 km of the river and are valued in commercial, sport, personal use, and subsistence fisheries (Healey 1991, Quinn 2005). Chinook salmon provide an important resource for communities living a subsistence lifestyle. Nearly 130,000 Alaskans live within the Yukon River drainage basin, and of those, 10 % in more than 60 Yukon River communities, rely on the river in part to supply food, water, and trade for their subsistence way of life (Brabets et al. 2000). In addition, the Chinook salmon commercial fishery often offers the only cash influx into lower Yukon River communities (Healey 1991, Quinn 2005).

Yukon River Chinook salmon begin to enter the river mouth soon after ice break-up at the beginning of summer, but the majority of the population delays their spawning migration until

mid-June (Evenson et al. 2009). Some Chinook salmon will begin spawning soon after river entry, but others may swim up to 3,200 km upstream to their final spawning grounds (Healey 1991). Usually 50 % of all migrating Chinook salmon spawn in Canadian waters (Evenson et al. 2009). Due to international migratory patterns of Yukon River Chinook salmon, biological escapement goals (BEGs) are especially important because they limit Alaska harvest and ensure that sufficient numbers of adult salmon reach the Canadian spawning grounds to maintain salmon population size (Yanagida 1987). To monitor up-river progress of returning adults, salmon are enumerated by counting towers, weirs, and sonar operations. This information is then used to determine if and when BEGs on tributaries have been met. Currently, BEGs only assess fish quantity, but have limited means of determining fitness of migrating fish. In addition, counts may be compromised by weather, water level, discharge, and overall poor visibility. This management tool also does not account for the quality of escapement onto the spawning grounds, e.g., sex ratio, size, and disease. These limitations can lead to inaccurate escapement estimates and uncertainties about spawning, thus raising questions about the status of the Chinook salmon population.

Reduced abundances of Arctic-Yukon-Kuskokwim (AYK) Chinook salmon stocks and recent reduced production has been a concern to commercial and subsistence fishers and fisheries managers. Chinook salmon returns in 2007, 2008 and 2009 were lower than predicted based on reported spawning escapements in the respective brood years (JTC 2011). In 2008, a commercial fishery for Chinook salmon on the Yukon River mainstem did not occur for the first time in almost a decade (JTC 2009), and the subsistence fishery was reduced by up to 50 % beginning in 2008 (JTC 2009, JTC 2011). In spite of these fishery adjustments, the interim management escapement target into Canada, as part of the U.S. Pacific Salmon Treaty obligations, in 2008 were not met. The 2009 management plan incorporated the protection of the first pulse of Chinook salmon entering the Yukon River, with early fish thought to contain mostly salmon headed to Canadian tributaries (Healey 1991). These restrictions harshly impacted the U.S. subsistence fishery along the Yukon River, but management actions were successful in meeting the interim management escapement goal into Canada in 2009 (JTC 2013). From 2008 to 2013, total numbers of returning Chinook salmon to the Yukon River have dropped from a historical average of around 300,000 fish to about half that number (JTC 2013, 2014). In both 2014 and 2015, BEGs into Canada were met (JTC 2014, 2015, ADF&G 2015). From 1994–2011, Chinook

salmon declines included a 12 % reduction in the subsistence and personal use fisheries, a 27 % decline in the commercial fishery, and a 21 % decline in the sport fishery (ADF&G 2013). These noticeable fluctuations in salmon abundance in recent years raise questions about the cause of the decline. Potential reasons for the decline in Chinook salmon returns to the Yukon River include factors such as bycatch by the walleye pollock *Gadus chalcogrammus* and other commercial fisheries, overfishing, targeting of larger and older fish, and reduced juvenile survival (Murphy et al. 2013). In addition, sustained high discharge in the Chena and Salcha rivers has been associated with low juvenile productivity by negatively affecting foraging conditions (Neuswanger et al. 2015). Another possible factor in these declines is the effect of disease, such as pathogen-induced mortality, reduced fecundity, or the inability of Chinook salmon to successfully migrate and spawn in tributaries.

Ichthyophonus hoferi (*Ichthyophonus* hereafter) is a marine-derived protozoan parasite infecting a wide range of marine and anadromous fish species, including salmonids (McVicar 1999, Kocan et al. 2004, Tierney and Farrell 2004, Gavryuseva 2007). Overall, more than 80 species of fishes are vulnerable to infection with *Ichthyophonus* (Daniel 1933, Gavryuseva 2007, Dykstra et al. 2013). The parasite was first reported in cultured brown trout *Salmo trutta* by Hofer (1893). *Ichthyophonus* is currently classified as a member of the mesomycetozoea, a monophyletic class of protozoans that includes several other pathogenic organisms (e.g., the rosette agent *Sphaerothecum destruens,* Arkush et al. 2003). Mass mortalities of Atlantic herring *Clupea harengus* have been attributed to *Ichthyophonus* epizootics in the mid-1990s (Mellergaard and Spanggaard 1997). *Ichthyophonus* prevalence in Pacific herring *Clupea pallasi* was around 30 % in the mid-1990's in Prince William Sound, Alaska, and caused high mortalities of up to 85 % (Marty et al. 1998). Similarly, Pacific halibut *Hippoglossus stenolepis* in the Bering Sea had an *Ichthyophonus* prevalence of over 30 % in 2012, while it was over 70 % in Prince William Sound, Alaska, which is indicative of an epizootic (Dykstra et al. 2013). Once infected with the parasite, fish are not believed to recuperate and most will ultimately perish from the disease (Sindermann 1965, Marty et al. 1998, Kocan et al. 1999, Hershberger et al. 2002). *Ichthyophonus* causes systemic infection which changes the tissues of infected organs, such as the heart and liver by necrotic reactions, thus disrupting normal organ function (Rahimian and Thulin 1996). This, along with starvation, seems to cause death of the host (Rahimian 1994). Internal lesions associated with *Ichthyophonus* infection are grossly visible and may include multifocal white

lesions on the heart, liver, spleen, and muscle of infected fish (McVicar 1982). The parasite can also form cysts in the muscle of the host (Daniel 1933). Progressive breakdown and inflammation of tissue surrounding the cysts can cause abscesses, an accumulation of white blood cells, which may range in size from microscopic to many inches (Daniel 1933). When the cysts burst, infected tissue is sloughed off, releasing the parasite into the environment. Planktonic feeders may ingest these infective cells, contracting the disease and serving as a source of infection to fish that ingest them (Kocan et al. 2010). *Ichthyophonus* is generally seen more frequently in Chinook salmon compared with other Pacific salmon species with up to 30 % prevalence in Chinook salmon (Kocan et al. 2004) vs. 10 % in coho salmon *Oncorhynchus kisutch* (Gavryuseva 2007). This is likely due to the extended marine residency of Chinook salmon of up to 8 years, and may also be associated with specific prey that they consume during their marine phase (Torgersen et al. 2002, Gregg et al. 2011). *Ichthyophonus* is likely an orally transmitted parasite through ingestion of infected prey, but is also presumed to be horizontally transferred (McVicar and Mackenzie 1972, Slocombe 1980, McVicar 1982, Okamoto et al. 1987, Sindermann and Chenoweth 1993, Gregg et al. 2012) or from exposure to ulcer-derived cells (Kocan et al. 2010). Infection may also result from simple co-habitation in the same environment, although laboratory infected herring did not infect unexposed fish via fish-to-fish transmission (Gregg et al. 2011, 2012). Juvenile Chinook salmon migrating out of the Yukon River are not infected with *Ichthyophonus,* however, infection occurs *prior* to when adults return to spawn (Kocan et al. 2004). This indicates that adults contract the parasite during the marine phase of their lifecycle.

The prevalence of *Ichthyophonus* in Yukon River Chinook salmon has varied substantially over the last 30 years (Kocan et al. 2004, Kocan et al. 2010, Horstmann-Dehn et al. 2012). While the cause of this variation is unknown, temperature changes in the Bering Sea may be a contributing factor. In the late 1990s, *Ichthyophonus* prevalence at the mouth of the Yukon River was about 17% (Kocan et al. 2004) and, over the next five years, infection prevalence increased to over 30 % (Kocan et al. 2004) before declining to about 10 % in 2010 (Zuray et al. 2012) (Figure 1.1). In the late 2000s, the average summer and winter water temperatures in the eastern Bering Sea were cold (NOAA mooring M2, NOAA's Pacific Marine Environmental Laboratory) and correspond with a noticeable drop in *Ichthyophonus* prevalence over the same time period (Figure 1.1, Horstmann-Dehn et al. 2012). It is possible that temperature changes in the Bering Sea affect the

composition and distribution of available prey; thus, the potential reservoir of *Ichthyophonus*-infected species that Chinook salmon feed on.

In poikilotherms, the inflammatory response is temperature dependent (Finn and Nielson 1971). In Chinook salmon, *Ichthyophonus* is likely activated from its dormant stage in infected fish by physiological and environmental triggers at the beginning of their migration (Rahimian 1998). Environmental variation can have direct and indirect impacts on the physiological reaction of fishes to an infection with a parasite or pathogen. Over the past 30 years, June water temperatures in the Yukon River have increased by 2.5 ºC (Figure 1.2). This change in in-river conditions is alarming, as a positive correlation was reported between *Ichthyophonus*-related mortality and water temperature along with an increase in fish mortality between 10 and 15 °C, with 100 % mortality between 15 and 20 °C (Okamoto et al. 1987). Similarly, Kocan et al. (2009) showed higher parasite load and faster die-offs with increases in temperature accompanied by reduced swimming performance in *Ichthyophonus*-infected rainbow trout *Oncorhynchus mykiss* between 15 and 20 °C.

The physiological effects of *Ichthyophonus* on fish succumbing to the disease, in particular the ability of salmon to successfully spawn, its effects on gamete quality, and potential second-generation effects on juvenile Chinook salmon survival, remain poorly understood. Fish egg and embryo vitality is linked to body condition of spawning females, and vitellogenesis is adversely affected by high levels of corticosteroids (Pankhurst and Van Der Kraak 2000, King et al. 2003). High concentrations of corticosteroids in turn are associated with disease, as well as increased water temperature (Strange et al. 1977, Olson 1986). Even healthy Yukon River Chinook salmon use virtually all of their substantial energy reserves to carry out one of the longest salmon migrations in the world. In diseased fish, energy demands are greater due to high physiological stress and costs linked with immune response (Kocan et al. 2006, Olson 1986, Vollenweider et al. 2011). Ichthyophoniasis is associated with reduced body reserves and emaciation in herring, in particular in colder waters (Rahimian 1998, Vollenweider et al. 2011). It is therefore possible that lipids may be re-routed from gonads of *Ichthyophonus*-infected Chinook salmon to complete the spawning migration, and then they either produce fewer or lower-quality eggs. Therefore, it is crucial to determine energy and lipid content of ova to establish if fish infected with the parasite (compared with healthy salmon) distribute the same energy stores to their eggs or consume

energy stored in gonads in addition to body lipid reserves to reach their final spawning grounds. Further, studies examining vitality, survival, and growth of alevins hatching from gametes of *Ichthyophonus*-infected parents are critically needed to foster pro-active management of all salmon life stages.

The goal of this thesis is to examine the effects of *Ichthyophonus* on spawning Chinook salmon and their offspring to assess potential impacts on reproductive success and effects on the second generation of Chinook salmon. The first chapter of my thesis investigates how proximate composition of muscle, eggs, and alevins may vary between *Ichthyophonus*-negative and *Ichthyophonus*-positive fish. The second chapter considers blood chemistry markers, and how an infection with *Ichthyophonus* may impact the comprehensive metabolic panel of Chinook salmon. I hypothesize that Chinook salmon infected with *Ichthyophonus* will use more energy reaching their spawning grounds, and therefore these fish will have lower egg survival to hatching and lower energy reserves compared to healthy fish. I further hypothesize that *Ichthyophonus*-infected fish and alevins produced by them will have higher levels of plasma cortisol and indicators of systemic inflammation compared to healthy fish.

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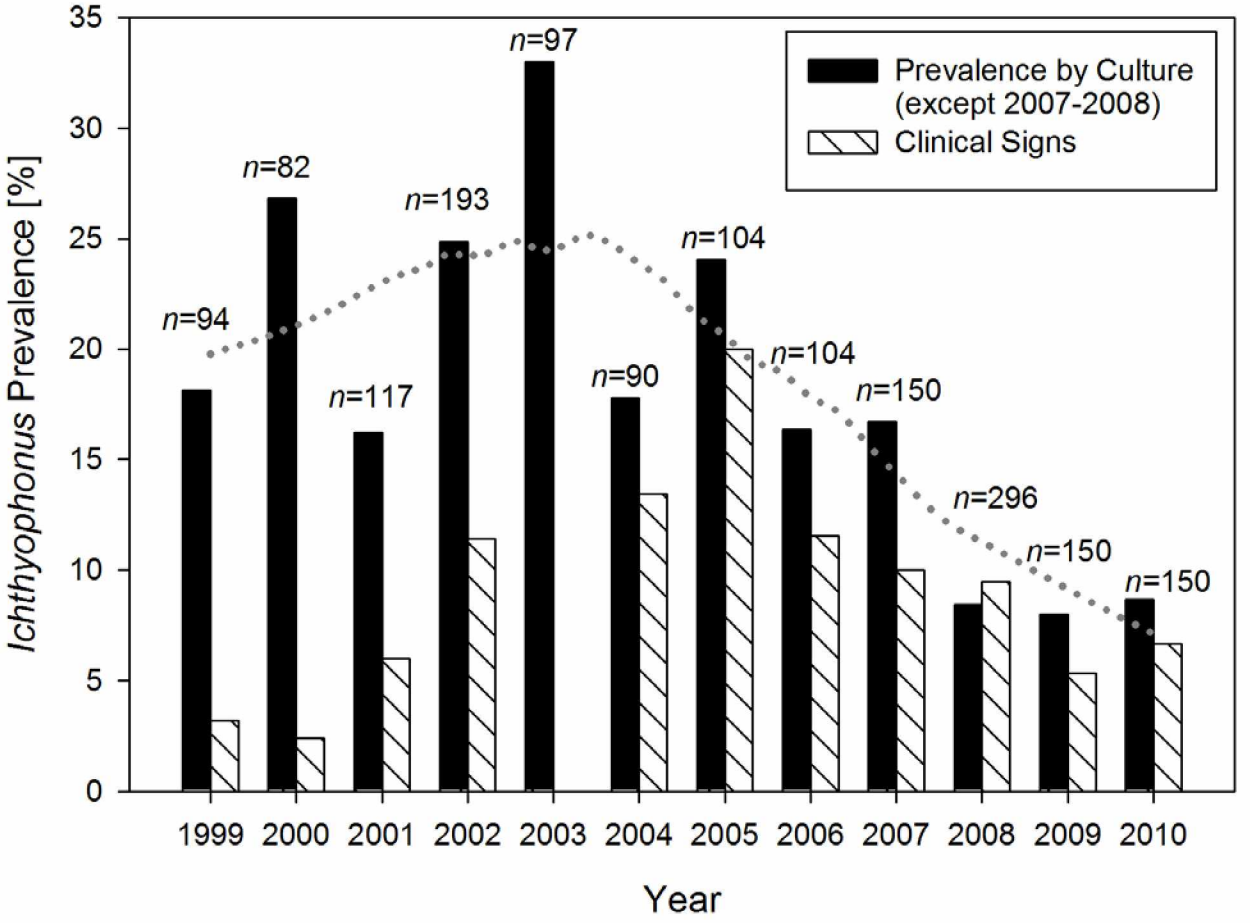

Figure 1.1: Time-series of *Ichthyophonus* prevalence. Time-series of *Ichthyophonus* prevalence at Emmonak, Alaska (Yukon River mouth) based on heart culture in Chinook salmon (*n* = sample size). LOESS non-parametric smoothing (dashed line) was applied to visualize temporal trends of parasite prevalence (courtesy of Horstmann-Dehn et al. 2012).

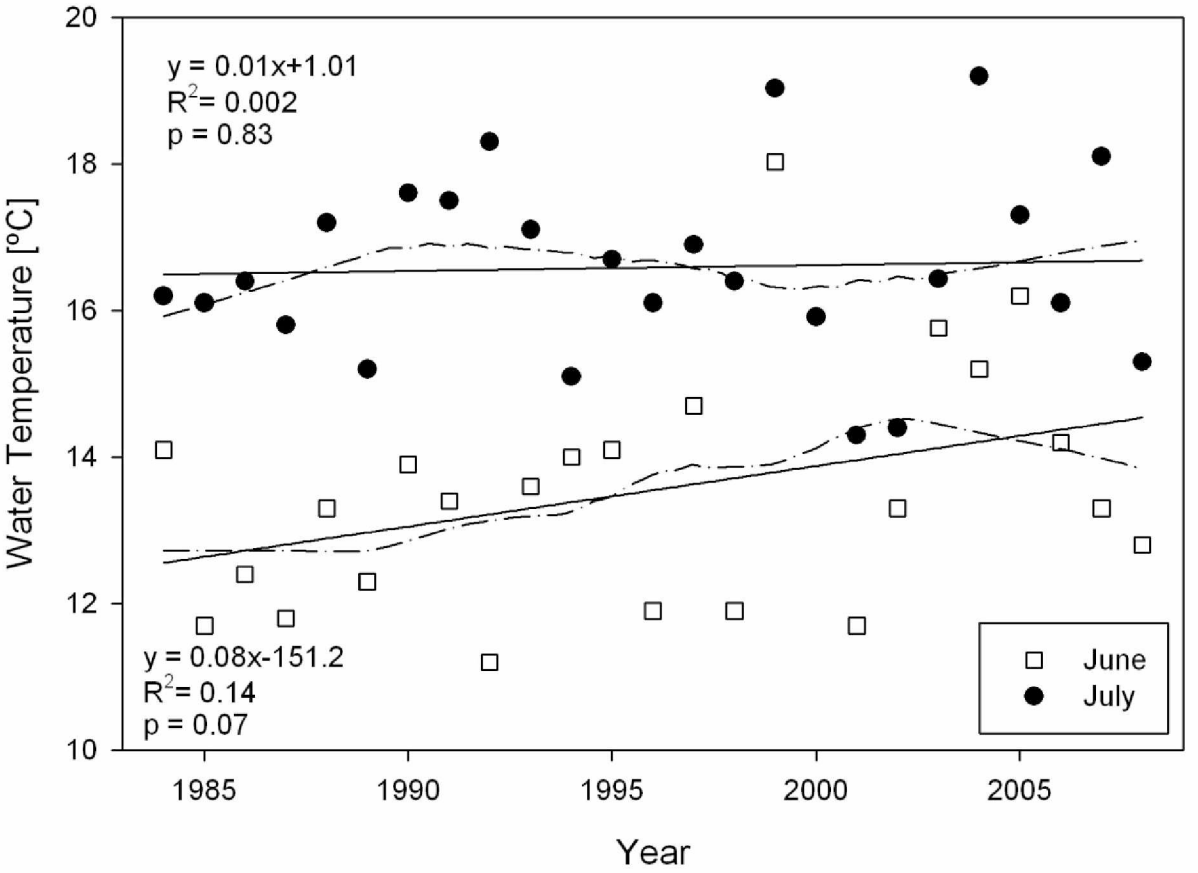

Figure 1.2: Historic water temperatures for the Yukon River. Historic water temperatures (means for June (□) and July (●)) measured at Alaska Department of Fish and Game test fishery projects for the Yukon River near Emmonak, Alaska. LOESS non-parametric smoothing (dashed line) was applied to visualize trends. Linear regression parameters are given in the graph (courtesy of L. Horstmann-Dehn).

Chapter 2: Effect of *Ichthyophonus* on spawning Chinook salmon and their offspring in a Yukon River tributary[1]

2.1 Abstract

In recent years, Chinook salmon *Oncorhynchus tshawytscha* returns to the Yukon River, Alaska, have substantially declined. In summer 2010 – 2012, spawning Chinook salmon (n=51, 32, and 12, respectively) were collected from the Salcha River, a tributary of the Yukon River, to determine the effects of *Ichthyophonus,* a protozoan parasite, on salmon reproductive success. Infection prevalence was 7.8, 6.3, and 8.3 % in 2010, 2011, and 2012, respectively. Eggs and milt from *Ichthyophonus*-infected and non-infected parents were collected in 2010 and cross-fertilized to investigate offspring survival and potential second generation effects induced by the parasite. No difference was found in sperm cell counts and the proportion of live sperm cells between *Ichthyophonus*-infected and non-infected males; the overall mean live cell proportion was 74.5±16.0 %. Egg lipid and water content was significantly higher in eggs from *Ichthyophonus*-infected females, while egg protein and energy content was not significantly different. Percent survival of fertilized eggs to hatching was not significantly different between offspring from *Ichthyophonus*-infected parents (Mean ± 1SD: 24.4±29.8 % survival) and non-infected parents (41.0±24.8 % survival). Proximate composition (%lipid, %protein, kJ/g) of muscle from spawning adult salmon also did not differ, nor did total body composition or morphology of alevins produced by either *Ichthyophonus*-infected or non-infected parents. Overall, infection with *Ichthyophonus* does not appear to impact the spawning ability or spawning success of Chinook salmon in the Salcha River, although other important determinants of gamete quality should be investigated in the future.

Key Words: *Ichthyophonus*, Chinook salmon, Yukon River, Salcha River, proximate composition, survival to hatching)

[1] Floyd-Rump TP, Horstmann-Dehn LA, Sutton TM, Skaugstad C. Effect of *Ichthyophonus* on spawning Chinook salmon and their offspring in a Yukon River tributary. In review with Diseases of Aquatic Organisms.

2.2 Introduction

Chinook salmon *Oncorhynchus tshawytscha* are valued in commercial, sport, and subsistence fisheries (Quinn 2005). These anadromous, semelparous fish return to freshwater habitats after spending on average 2 to 4 years at sea (Allen and Hassler 1986, Healey 1991). A small percentage of the male population may return to freshwater to spawn after only one year at sea (Healey 1991). Larger Chinook salmon require more time to return to their natal spawning grounds, and this extended phase in the ocean allows them to increase their size by increasing prey consumption (Healey 1991). Yukon River Chinook salmon return after two to six years (Evenson et al. 2009). Spawning can occur in coastal regions (Behnke 2002), and some stocks undergo extensive migrations, e.g., over 3,200 km upstream in the Yukon River (Healey 1991).

Chinook salmon provide an important resource for subsistence communities along the Yukon River (Busher et al. 2009). Nearly 130,000 people live in the Yukon River drainage basin, and approximately 10 % of them rely at least partially on the river to supply food, water, and resources for trade (Brabets et al. 2000). Chinook salmon are highly valued due to their high oil content (Gilbert 1922, Mink 2013). These lipid deposits serve as energy resources for Chinook salmon, enabling them to complete long migrations without consuming food, while still being able to spawn when they reach their spawning grounds (Gilbert 1922).

The mainstem of the Yukon River covers over 3,200 km flowing from headwaters in British Columbia, Canada, through the Yukon Territory, then Alaska before draining into the Bering Sea. Including its tributaries, the Yukon River is the fourth largest drainage basin in North America (Ge et al. 2013). The Tanana River and its clear-water tributaries, the Chena and Salcha rivers, are some of the largest tributaries and producers for Chinook salmon of the Yukon River in interior Alaska. About half of all production of these fish occurs in Canadian waters (Brabets et al. 2000), while the majority of the harvest occurs in Alaska (Templin et al. 2005). Chinook salmon harvest is highly regulated by international treaties with input from multiple federal, state, and Native/First Nation groups, as well as other stakeholders and non-governmental organizations (Brabets et al. 2000). One of the main governing treaties is the Pacific Salmon Treaty (Yanagida 1987), which sets salmon harvest regulations established on evaluation of run strength and total allowable catch for Chinook salmon into the Canada mainstem of the Yukon River (Hayes et al. 2014). Subsistence fishing takes the highest priority in both Alaska and Canada, giving

subsistence fishing rights before both commercial and sport fishing (Shelden et al. 2014). Annual decisions on commercial and sport Chinook salmon harvests are determined using data from biological escapement goals (BEGs) and interim management escapement goals (IMEGs) to determine if treaty obligations have been met. The BEGs are a tool used to manage stocks of concern (such as Chinook salmon) and provide the greatest potential for meeting maximum sustainable yield objectives (Conitz et al. 2012). As Chinook salmon migrate up the Yukon River and tributaries to their spawning grounds, they are enumerated by counting towers, weirs, and sonar operations (e.g., Andreafsky River weir, Salcha River tower, Eagle sonar). At the end of the Chinook salmon run, the Alaska Department of Fish and Game (ADF&G) initiates carcass collections, which provide information about run quality, such as age, sex, and length of fish, but not disease. While these mechanisms allow managers to estimate the number of Chinook salmon traveling upstream, it provides no information about disease status or body condition, which can impact numbers of offspring and juvenile survival.

Chinook salmon in Arctic-Yukon-Kuskokwim watersheds have undergone striking reductions in abundance beginning in the late 1990s (Schindler et al. 2013). Chinook salmon are essential to subsistence, commercial, personal use, and sport fisheries throughout Alaska, and recent declines in productivity and abundance have led to social and economic hardships in rural and urban Alaska (ADF&G 2013). Substantial harvest constraints have been implemented in reaction to reduced Chinook salmon returns throughout the Yukon River, which involves the closure of commercial fisheries and decreases in subsistence fisheries (Murphy et al. 2013). In 2014, Yukon River commercial, sport, and even subsistence Chinook salmon fisheries were closed (ADF&G 2014). Yukon River Chinook salmon populations have declined by almost 60 % from 2003 – 2010, most likely due to anthropogenic and environmental stressors (Zuray et al. 2012).

In the mid-1980s, *Ichthyophonus hoferi* (*Ichthyophonus* hereafter) was first identified in Chinook salmon in the Yukon River by the ADF&G and U.S. Fish and Wildlife Service after fishers noted an increasing number of harvested fish with white pustules on heart and skeletal muscle (Kocan et al. 2004a). These fishers also noticed an unpleasant fruity odor when infected fish were processed (Kocan et al. 2003, 2004a). *Ichthyophonus* is a marine-derived parasitic protozoan with low host specificity that affects over 80 species of marine and anadromous fishes, including salmonids (Olson 1986, McVicar 1999, Mendoza et al. 2002, Kocan et al. 2004a). When juvenile

salmon migrate out of the Yukon River, they do not appear to be infected with *Ichthyophonus* (Kocan et al. 2004b). However, when adults return to spawn, a portion of the population is infected indicating adults contract the parasite during the marine phase of their lifecycle (Kocan et al. 2003, Arkoosh et al. 2004).

Ichthyophonus is likely an orally transmitted parasite (Slocombe 1980), but it may also be horizontally transferred (Hershberger et al. 2008, Kocan et al. 2010). Horizontal transfer occurs when a viable resting spore of *Ichthyophonus* is consumed by a fish and enters the digestive system (Vollenweider et al. 2011). Once the spore reaches the stomach, the low pH causes it to germinate (McVicar 1999). As hyphae grow, they puncture the gastrointestinal lining. When spores reach the neutral pH of the blood, the hyphae release more spores into the bloodstream, where they proliferate in the organs and muscle tissue of the organism (McVicar 1999). The *Ichthyophonus* lifecycle may also involve a combination of both transmission mechanisms, i.e., oral and horizontal. *Ichthyophonus* is not transmittable to mammals; the survival of *Ichthyophonus* is less than three minutes at mammalian body temperatures of around 40 °C (Spanggaard and Huss 1996). *Ichthyophonus* is potentially transmitted through an intermediate host, and the copepod *Calanus finmarchicus* has been positively identified with an infection (Wolke 1975). Torgersen et al. (2002) also reported an *Ichthyophonus*-like pathogen in *Calanus* spp. that changes the coloration of the infected copepod, and consequently make it an easy prey target. Marine-phase Chinook salmon are primarily piscivorous and prefer seasonally abundant Pacific herring *Clupea pallasii* (Brodeur et al. 1992). *Ichthyophonus* prevalence in Pacific herring was around 30 % in the Bering Sea during the mid-1990s (Marty et al. 1998). In 2002, Pacific herring prevalence ranged from 12 % to nearly 60 % with the incident of *Ichthyophonus* increasing with age (Hershberger et al. 2002). In the early 1990s, there were mass mortalities along the north Atlantic coast caused by *Ichthyophonus* (Rahimian and Thulin 1996). Herring, in turn, rely heavily on calanoid copepods (Foy and Norcross 1999); thus, the lifecycle of *Ichthyophonus* leading to Chinook salmon could be complex. In the early 1990s, *Ichthyophonus* was found in 2 % of walleye pollock *Gadus chalcogrammus* (Eaton et al. 1991); by the end of the 2000s, prevalence had reached over 25 % in Alaskan pollock and walleye pollock has been proposed as a reservoir species (White et al. 2014). *Ichthyophonus* is a metropolitan parasite that infects many different species of fish and has the potential to cause further epizootics in the future.

Water temperature influences physiological processes, including inflammation and stress response, in poikilotherm organisms (Finn and Nielson 1971, Strange et al. 1977). Increased *Ichthyophonus*-related mortality and higher parasite loads have been described in salmonids at temperatures above 10 °C (Okamoto et al. 1987, Kocan et al. 2009). In-river conditions in the Yukon River have changed over the past 30 years, with June water temperatures having increased by approximately 2.5 ° C and summer water temperatures readily approaching 15 °C (Kocan et al. 2004a, 2009, Kahler et al. 2007). Similarly, Bering Sea water temperatures have undergone marked changes over the last 30 years, which has affected fish movements, abundance, and disease dynamics (Overland and Stabeno 2004, Brierley and Kingsford 2009). If water temperatures continue to rise, it could cause increased stress in Chinook salmon, which can cause decreased immune response, in addition to the parasite proliferating at a higher rate at warmer temperatures (Kocan et al. 2009). The combined effects could be disastrous to this already struggling population. Understanding changes in disease prevalence as well as disease effects in declining Chinook salmon stocks is therefore important.

Infection with *Ichthyophonus* can result in external and internal tissue damage and can cause substantial changes in physiological function of infected organisms. Large-scale necrosis in multiple tissues, including muscle, heart, liver, and kidney, can contribute to organ failure and pre-spawning mortality in salmonids and other species (Kocan and Hershberger 2006, Dykstra et al. 2013). *Ichthyophonus* has caused major recurring epizootics and mass die-offs in Atlantic herring *Clupea harengus* (Rahimian and Thulin 1996, Kramer-Schadt et al. 2010). Infection with *Ichthyophonus* has further been associated with reduced body reserves and emaciation in the fish host (Rahimian 1998, Kramer-Schadt et al. 2010). Juvenile Pacific herring infected with *Ichthyophonus* also showed substantial depletion of energy reserves (Vollenweider et al. 2011). Rainbow trout *Oncorhynchus mykiss* infected with *Ichthyophonus* exhibited a significant reduction in hematocrit, thus contributing to reduced swimming performance and stamina (Rand and Cone 1990). Further, oxygen uptake after exercise was lower in salmon infected with *Ichthyophonus* relative to non-infected fish (Tierney and Farrell 2004). It is therefore essential to study physiological condition of salmon and their offspring, particularly in a species that depends heavily on lipid reserves and stamina during a long migration, such as Yukon River Chinook salmon.

The aim of this study was to determine the effects of *Ichthyophonus* on Chinook salmon reproductive success. Gamete quality (sperm counts and energy allocation to eggs) as well as adult body proximate composition was determined for *Ichthyophonus*-infected and non-infected Chinook salmon. Hatching success, growth, and energy stores of alevins were determined by comparing the fertilized gametes of *Ichthyophonus*-infected parents to non-infected controls. We hypothesized that gametes of *Ichthyophonus*-infected salmon would be lower quality, and that resulting offspring would have lower hatching success and available resources for growth than those of non-infected fish.

2.3 Methods

2.3.1 Field Sampling and Egg Collection

In 2010 and 2011, Chinook salmon were sampled from the Salcha River approximately 39 km upstream at the following coordinates: 64°47'N, 146°97'W. In summer 2010, we collected 51 Chinook salmon (27 females [including 3 freshly dead] and 24 males) from July 29th to August 1st. On July 19th 2011, we sampled 32 Chinook salmon (15 females and 17 males). Salmon sampled in 2010 and 2011 were captured by boat electrofishing. On July 26th 2012, we sampled 12 Chinook salmon (6 females and 6 males) in collaboration with ADF&G at the Salcha River boat ramp at the following coordinates: 64°46'N, 146°96'W. These fish were sampled using rod and reel sport fishing gear. Changes in sampling procedures and sample sizes were unavoidable, as all collections were dependent on ADF&G approval after evaluation of run strength and BEG to the river system. In 2011 and 2012, we opportunistically sampled Chinook salmon being utilized for ADF&G hatchery broodstock use, and not solely for the purpose of this project.

Once captured, fish were transferred into net pens, approximately 1.2 m x 1.2 m x 2.4 m in size with a 3.8 cm-mesh. Holding pens were placed in the river current for continuous water flow. Fish were held at a maximum density of 12 fish per pen, with males and females kept separated. Although males were sexually mature as soon as they reach the spawning grounds, females were at varying stages of maturity when captured. Therefore, holding time in the pens varied, but was generally 1 to 7 days until females were sexually mature enough to extract eggs. Maturity of females was characterized by loss of skein structure and was determined by gentle palpation of the abdomen and discharge of eggs through the ovipositor.

Sexually mature adult fish were euthanized by cranial concussion, and morphometrics (i.e., girth and total length) were measured using a flexible measuring tape to the nearest 5 mm for total length and to the nearest 1 mm for girth (Table 2.1). Total length was measured from the tip of the nose to the fork of the tail, and girth was measured directly anterior to the dorsal fin. Eggs from each female were then harvested by opening the abdomen with a sterile razor blade and gently releasing the eggs into an 18.9 L bucket. Approximately 10 mL of milt from each male was collected in sterile weigh boats, which was expelled by applying gentle pressure to both sides of the abdomen. Buckets and weigh boats holding eggs and milt were placed in flowing river water to keep cool and out of direct sunlight until ready for fertilization.

After gametes were removed, fish were then examined internally to determine if lesions consistent with *Ichthyophonus* infection were grossly visible on the heart (Figure 2.1a). Clinical signs of infection with *Ichthyophonus* include white nodular lesions on the heart or other highly vascularized tissues (Figure 2.1a). After collection, cardiac muscle was cultured to determine if live spores were present (details below, Figure 2.1b). Once gross infection status was determined, four treatment groups were developed for cross-fertilization experiments: 1) *Ichthyophonus*-infected male x non-infected female; 2) non-infected male x *Ichthyophonus*-infected female; 3) non-infected male x non-infected female; and 4) *Ichthyophonus*-infected male x *Ichthyophonus*-infected female. As the infection status of the fish was not known *prior* to gamete removal and fertilization of gametes had to take place within 10 minutes *post mortem*, we were not able to create treatment group 4. Eggs were placed in clean, dry 3.8-L containers, and milt was added with flowing river water for fertilization. After fertilization, eggs were left in coolers for at least one hour to allow eggs to water harden for subsequent transportation of fertilized eggs.

Fertilized eggs were transported directly to the University of Alaska Fairbanks (UAF) hatchery facility. At the facility, fertilized eggs were treated with a 10 % iodine solution to reduce fungal and bacterial growth and placed into heath incubation trays (45.7 cm L x 35.6 cm W x 7.6 cm D). A MariSource vertical incubation system for salmon with 16 heath trays was used, and each tray had the capacity to hold up to 10,000 eggs. Eggs were kept in the dark almost constantly, as light can harm developing eggs (Browman et al. 2003). Heath trays were covered completely with black plastic to reduce overhead light. Dead eggs were identified by a change in color, from a deep orange to a white or light orange color. Nonviable eggs were removed and counted daily,

and egg removal reduced the amount of bacteria and fungi in the system (Heikkinen et al. 2013). The hatchery system was checked daily for water temperature and water-quality parameters (see below for details). Once eggs reached the eyed stage (at about 4 weeks of development), they were shocked *via* agitation by transferring eggs within a tray into a clean 189.3-L container with about 40 L of chilled system water in the bottom (Dwyer et al. 1993). Shocking eggs allowed for the identification of dead eggs by rupturing the yolk and turning them white (Leitritz and Lewis 1980). Eggs hatched approximately 3 weeks after the eyed stage was reached. About three weeks after egg hatch, 10-15 g of alevins from each of the 16 heath trays were sampled for morphometric measurements and proximate composition analyses. Fifteen individual alevins from each of the 16 heath trays were weighed to the nearest 0.0001 g, and total length and height of the yolk sac were measured to the nearest 1 mm using digital calipers.

In 2011 and 2012, samples were collected in collaboration with the ADF&G hatchery, and parentage of each fertilization pairing was tracked by the hatchery. During these two years, ADF&G fertilized one male with one female, without the multiple crossing we targeted in 2010. In 2011 and 2012, the proximate composition of alevin from the ADF&G hatchery was analyzed, but alevin morphometrics were not measured as freeze-thawing compromised their membrane/structural integrity.

2.3.2 Hatchery

The hatchery system at UAF consisted of a MariSource vertical incubator with 16 hatching trays on top of a 492.1-L raceway. The flow rate for the system was 22.7 L per minute, and the system was closed with two 196.8-L water reservoirs and one submersible chiller unit (Frigid Units, Inc., Toledo, Ohio) in each water reservoir to maintain optimal chilled water (10 °C) for the developing eggs. The water in the system had been filtered using reverse osmosis; therefore, Instant Ocean™ was required to add minerals (hardness) as well as ions for conductivity and salinity. Water quality (i.e., alkalinity, ammonia, nitrite, pH, and total hardness) was monitored using a Nine-Parameter Test Kit (Hach, Model FF-1A). In addition, dissolved oxygen, conductivity, salinity, and temperature were measured using a YSI Model 85 Handheld Oxygen, Conductivity, Salinity, and Temperature System (Model 451-85). All water-quality parameters were assessed daily (Table 2.2). The hatchery system was only set up about one week *prior* to egg collections, and there was not adequate time for the bacteria in the biofilter to proliferate and

reduce the ammonia levels in the hatchery. Ammonia levels approaching 2.0 mg/L can be toxic to fish (Leitritz and Lewis 1980). Thus, weekly water changes were necessary to manually reduce ammonia to acceptable levels for the developing eggs.

2.3.3 Ichthyophonus Detection

For each adult fish, the heart, liver, spleen, and posterior kidney were examined for grossly visible lesions consistent with *Ichthyophonus* infection (Figure 2.1a). *Prior* to cutting open the fish to obtain tissue samples, mucus and blood were wiped from the ventral surface using a clean paper towel. The skin was incised along the ventral midline from the vent to the operculum with a sterile razor blade to expose the heart, taking care to keep the pericardium intact to avoid contamination. Approximately 1 g of cardiac muscle was removed with a second sterile blade. The tissue was cut in two sub-samples in a sterile weigh boat; one sub-sample was placed into Eagle's minimum essential medium (MEM-5) supplemented with 5 % fetal bovine serum, and 100 µg/mL of each penicillin, streptomycin, and gentamycin (Kocan and Hershberger 2006). The tissue was refrigerated at 4 °C until shipment to the State University of New York, Syracuse, NY, for *Ichthyophonus* culture, typically within 48 hours. There, the heart sample was incubated at 14 °C and examined daily microscopically for up to 14 days for the presence of *Ichthyophonus* spores to confirm clinical infection (Figure 2.1b). A second sub-sample of cardiac muscle was placed in 95 % ethanol and shipped to Purdue University, West Lafayette, IN, for molecular confirmation of infection. The presence of *Ichthyophonus* 18S rDNA was evaluated using polymerase chain reaction (PCR) following the procedure described by Whipps et al. (2005).

2.3.4 Proximate Composition Analysis

Sub-samples of muscle from adults, unfertilized eggs, and alevins were sampled for determination of proximate composition (% lipid, % crude protein, and energy content) at the Marine Mammal Ecophysiology Lab at UAF. Wet tissue mass of muscle, eggs, and alevins was determined using a micro-balance (Sartorius Model MP2; 0.0001 g), and samples were freeze dried (VirTis Sentry) for a minimum of 48 hours. Water content of all tissues was determined as loss of mass during the freeze-drying procedure. Approximately 0.5 g, measured to the nearest 0.0001 g, of dried tissue was placed into a cellulose thimble and then lipid-extracted using chloroform:methanol (2:1) in a modified Soxhlet procedure after Schlechtriem et al. (2003). Samples were

continuously flushed in the Soxhlet with clean chloroform:methanol for 24 hours. Percent lipid in the sample was determined gravimetrically. All samples were analyzed in duplicate, and the mean of duplicate samples was reported. If there was more than a 20 % difference in values between duplicates, the sample was run again in duplicate. Tissue nitrogen content was measured using a Leco Nitrogen/Protein Analyzer (Leco-FP-528) with 950 °C combustion temperature. An EDTA standard was run twice after every 15 samples. Instrument error based on repeat analyses (n=32) of the standard was ±0.4 %. Ash content refers to the mineral content of a sample. Ash content of the sample was determined *via* combustion at 850 °C for 8 hours in a muffle furnace. Approximately 0.5 g of dried sample (in duplicate) was weighed to the nearest 0.0001 g into a porcelain crucible and combusted; the remaining ash was again weighed. Subtractions of ash content from dry matter allowed for calculation of organic matter in the sample, and further subtraction of lipid provided lean dry mass. Crude protein was calculated from nitrogen content of lean dry mass, assuming all nitrogen was bound to protein (Paolini et al. 2006). Energy content of tissues was determined using bomb calorimetry (Parr Model 6300). Lyophilized tissue was formed into pellets and analyzed in duplicates in a pressurized oxygen atmosphere. The average mass of a pellet was approximately 0.5 to 1 g of sample to assure that the temperature increase in the water jacket did not surpass the range of the thermometer and to provide a safe level of combustion. After combustion, the temperature increase in the 2-L water jacket around the stainless steel bomb was determined and used to calculate the energy content of the sample. Up to 5 benzoic acid standards were analyzed at the beginning of each analytical day to calibrate the instrument, and then the standard was run every 10 samples. Instrument error for the bomb calorimeter was ±0.003 kJ/g based on 36 measurements of the standard. Results were reported as dry tissue weight unless otherwise noted.

2.3.5 Sperm Counts

Milt was removed from spawning Chinook salmon males by gently palpating the abdomen until milt flowed freely. A sub-sample of milt was placed in HEPES-buffer (dilution 1:1000) and stored at 4 °C until analysis at UAF. The solution contained 10 mM HEPES, 150 mM NaCl, and 10 % Bovine Serum Albumin with the pH adjusted to 7.4. This buffer is used to maintain physiological pH balance, even when CO_2 concentrations (and consequently pH) change due to sperm cell metabolism. The total number of sperm cells per mL was determined using InCyto C-

Chip disposable hemocytometers (10x10 grid, each square was 0.2 mm^2). Milt samples were diluted to 1:1000, and then 10 µL was pipetted into each chamber of the hemocytometer and then counted by microscope. Total cell counts were done all three sampling years at 40x magnification on a Leica DM 1000 microscope, and all 100 squares were counted for each sample at a dilution of 1000x. In addition, a live/dead sperm viability kit (Molecular Probes, MP 07011) was used in 2011 and 2012 to analyze the viability and fertilizing capability of the collected sperm following kit instructions. This method was added after the 2010 field season, so data are only available for 2011 and 2012 samples. This fluorescence-based assay has a membrane-permanent nucleic acid dye (SYBR 14) that stains live cells a bright green color. A standard dead cell stain, propidium iodide, was used to mark dead cells in the sample (which appeared bright red). Both SYBR 14 dye and propidium iodide can be excited using visible-wavelength light. When these dyes are bound to cells with DNA, the fluorescence emission is between 516 nm for green light and 617 nm for red light. To determine live/dead cell counts of sperm, stained sperm cells at 1000x dilution were pipetted into the viewing area of the hemocytometer (as described above). The grid was observed and live (green) and dead (red) sperm cells counted in 100 squares using 40x magnification on a Leica DM 1000 microscope equipped with an ultraviolet filter (OMAX UV-V filter). The milt sample from one *Ichthyophonus*-infected fish sampled in 2010 was lost in a refrigerator malfunction.

2.3.6 Statistical Analyses

Standard descriptive statistics were compiled, i.e., mean, median, standard deviation, and range, for hatching success, proximate composition of eggs, alevins, and adults, and sperm counts. The statistical program package R version 3.0.2 was used for all statistical tests, with an alpha of 0.05 considered significant (R Development Core Team 2008). Within R, a Shapiro-Wilk's normality test and Bartlett's test for equal variances was performed to assess if data met assumptions for ANOVA. A Tukey pairwise comparison was performed following an ANOVA to determine if the above parameters were different between infected and non-infected fish and their offspring. A Kruskal-Wallis test was used for data that violated normality and homoscedasticity assumptions, such as adult muscle % lipid, % protein, and energy content. Principal component analysis (PCA) is a statistical method used to highlight variation and bring out strong patterns in a dataset (Quinn and Keough 2002). Principle components analysis was performed on all

proximate composition parameters for eggs, adult muscle, and alevins using PRIMER-E (Version 6.1.16) to assess the combined effects of all variables when comparing *Ichthyophonus*-infected and non-infected fish and their offspring. In addition, multidimensional scaling on a Bray-Curtis similarity matrix without data transformation was performed on proximate composition parameters for Chinook salmon eggs, adult muscle, and alevins using PRIMER-E (stress of 0.10 is fair and >0.2 is poor, Kruskal 1964).

2.4 Results

Prevalence of *Ichthyophonus* in the Salcha River was 7.8 % (3 females, 1 male) in 2010, 6.3 % (2 males) in 2011, and 8.3 % (1 male) in 2012. Infection with *Ichthyophonus* was detected by culture and then confirmed with PCR analysis, and there were no false positives or false negatives. However, there was one individual in 2010 that displayed clinical signs of *Ichthyophonus*, but was not positive by either culture or PCR. In 2010, the mean %survival (±1SD) of eggs to hatching for offspring of *Ichthyophonus*-infected parents was 24.4±29.8 % (n=8), and the mean % survival of eggs to hatching for non-infected parents was 41.0±24.8 % (n=8) (Table 2.1). There was no significant difference between eggs from *Ichthyophonus*-infected fish compared with non-infected fish (ANOVA: F=1.50, p=0.25). Egg survival for gametes fertilized by an *Ichthyophonus*-infected male was 29.49±41.60 % (n=2 crosses), gametes from *Ichthyophonus*-infected female's fertilized non-infected males was 11.26±15.92 % (n=6 crosses), compared with 58.87±1.39 % (n=8 crosses) for eggs from non-infected females fertilized by non-infected males. Survival among trays was highly variable (as attested by large standard deviations), but overall survival was not significantly different between offspring from non-infected and *Ichthyophonus*-infected parents (F=1.50, p=0.25)

There was a significant difference between sperm cell counts for the three sampling years (ANOVA, F=48.50, p<0.0001). Sampling years 2011 and 2012 were significantly different from 2010 (p=0.0001), but not from each other (p>0.05). Mean sperm cell counts for non-infected Chinook salmon in 2010 were 6.9±3.8 x 10^9 cells/mL (n=25), 14.0±8.1 x 10^9 cells/mL in 2011 (n=15), and 18.1±1.7 x 10^9 cells/mL (n=6) in 2012. There was no milt from an *Ichthyophonus*-infected Chinook salmon in 2010 due to a refrigerator malfunction; therefore, no comparison was made, and there was no difference in milt counts between *Ichthyophonus*-infected and non-infected males found in 2011 (F=0.60 p=0.40) or 2012 (F=0.50 p=0.60) using a one-way

ANOVA. Mean sperm cell counts for *Ichthyophonus*-infected Chinook salmon were 14.3±3.6 x 10^9 cells/mL (n=2) in 2011 and 12.6 x 10^9 cells/mL in 2012. The proportion of live sperm cells in non-infected salmon was 70.2±18.1 % in 2011 and 79.9±14.7 % in 2012. The live cell count was not available for 2010. The proportion of live cells in *Ichthyophonus*-infected salmon was 75.4±15.8 % in 2011, and 77.8 % in 2012, and it was not different from non-infected fish (p>0.05). There was no correlation between male salmon total length and sperm cell counts (p>0.05).

Proximate composition of adult salmon (% lipid, % crude protein, and energy content) did not differ between *Ichthyophonus*-infected and non-infected fish (Kruskal-Wallis test, p>0.05; Table 2.3). However, egg proximate composition showed some differences. The fat content of eggs from infected Chinook salmon showed unexpectedly higher % lipid (ANOVA, F=6.00, p=0.02) and higher %water (F=8.70, p=0.005) relative to eggs from non-infected females (Table 2.4). Egg lipid and water content was approximately 7.4 and 4.4 % higher, respectively, in infected salmon. Egg energy content and protein content was non-normally distributed with unequal variance; therefore, a Kruskal-Wallis test was used. Egg energy content was not significantly different (H=4.00, p =0.05) between eggs from *Ichthyophonus*-infected Chinook salmon and eggs from non-infected females. Egg protein content was not significantly different (H=0.23, p=0.41 between eggs from *Ichthyophonus*-infected Chinook salmon and eggs from non-infected females.

Once eggs hatched into alevins, all analyzed proximate composition parameters (% lipid, % crude protein, and energy content; Table 2.5), as well as morphometrics (i.e., weight, length, and yolk sac height; Table 2.6), were similar between offspring from non-infected and *Ichthyophonus*-infected parents (p>0.05 for all parameters). Principal component analysis (PCA) supported the overall lack of differences between *Ichthyophonus*-infected and non-infected adult salmon and their offspring. The first two principal components (PC) using proximate composition parameters of spawning Chinook salmon explained 81.3 % of the variability, with little separation between the two groups (Figure 2.2). In contrast, proximate composition parameters of eggs showed some separation in PC1 with eggs from *Ichthyophonus*-infected females having a higher positive loading in %lipid, agreeing with results of the ANOVA (Figure 2.3). Total variability explained by the first two PCs was 87.3 % (Figure 2.3). The PCA of proximate composition of alevins showed no separation of the two groups, while PC1 explained 62.3 % of variability and PC2

explained 27.5 % for a cumulative 89.8 % (Figure 2.4). Multidimensional scaling on a Bray-Curtis similarity matrix without data transformation on proximate composition parameters for adults, alevins, and eggs also showed no underlying structure (stress=0.17, 0.14, and 0.11 for adults, alevins, and eggs, respectively).

2.5 Discussion

Yukon River Chinook salmon undergo one of the longest migrations in the world to reach their spawning grounds (Healey 1991). To complete this migration, they must acquire large energy reserves before beginning their freshwater journey where they do not consume food (Crossin et al. 2004). Chinook salmon may lose up to 99 % of their lipid stores over the course of spawning activities (Love 1970, Shearer 1993). Columbia River Chinook salmon have a dry muscle lipid content of about 19 % at river entry, and lipid reserves were reduced to about 16 % when they reached their spawning grounds 480 km upstream (Mesa and Magie 2006). Lipid content of Chinook salmon sampled in the Yukon River mainstem near the Rampart Rapids (1200 km up river) was between 35-40 % (dry mass) using bioelectrical impedance and proximate composition analyses (Margraf et al. 2005) and was at the lower range of body lipid stores compared with other salmonid species (Hartman and Margraf 2008). In the current study, Chinook salmon on their spawning grounds had on average of 20 % dry lipid content in muscle, which is lower than the range of Chinook salmon muscle in the Yukon River mainstem (Margraf et al. 2005). This is to be expected for fish on the terminal spawning ground, and we did not find a difference between *Ichthyophonus*-infected and non-infected fish. In general, fish that are stressed or diseased have higher energetic demands due to the immune response brought on by disease, in addition to normal body functions (King et al. 2003, Rand et al. 2006). However, the effects of the parasite on proximate composition may be subtle when compared with the drastic changes undergone during salmon migration and spawning. While migrating upstream, the two greatest somatic energy needs of female salmon are swimming and gonad maturation (Rand and Hinch 1998). Male salmon on the other hand use reserves for active metabolism and development of secondary sexual characteristics (Kinnison et al. 2003). Amidst all of these changes, it is conceivable that the effect of disease on proximate composition is not strong enough to impact the already high lipid content of muscle, and samples for this study were obtained at the end point of spawning. It

is also possible that the small sample sizes in our study were not sufficient to see an effect of disease on proximate composition.

Proximate composition factors, such as protein, water, and energy content, may be affected by migration as well. Spawning salmon may exhaust up to 72 % of their protein stores during reproductive activities (Love 1970, Shearer 1993). Total moisture is inversely associated with lipid content and increases or decreases as lipid is consumed or deposited (Shearer 1993); therefore, water content is highly correlated with all proximate composition components (Jonsson et al. 1997). Sockeye salmon *Oncorhynchus nerka* migrating up the Fraser River in British Columbia, Canada, lost 6 % of their lipid content and gained 11 % moisture content (Idler and Bitners 1958). Spawning Atlantic salmon *Salmo salar* showed an energy loss of 60-70 % over the course of spawning migration (Jonsson et al. 1997). In the current study, we did not see any significant differences between *Ichthyophonus*-infected and non-infected Chinook salmon which were sampled at their terminal spawning grounds. The lack of differences could be attributed to the long migration that the fish have undergone, where they utilized the majority of their body resources to reach their final destination.

Body condition of spawning female fish can affect hatching success and survival of offspring (Berkeley et al. 2004). Body condition is determined by the physiological state, energetic needs, and availability of resources. Biological condition of the female determines the condition and number of eggs produced (Kamler 2005, Muir et al. 2014). For example, poor condition females, with low lipid content and high water content in muscle tissue, will generate a limited amount of low quality eggs that have small size, increased water content, and low lipid content (Kamler 2005, Muir et al. 2014). Nutrition of the female is an essential aspect in determining fecundity, gametogenesis, and gamete quality, including egg size, alevin size, reproductive timing, and reproductive hormones (Kjesbu et al. 1991, Bobe and Labbe 2010, Burton et al. 2013). Pacific herring that were infected with *Ichthyophonus* showed a 30 % reduction in total stored energy, with the greatest impacts of infection occurring at temperatures of less than 10 °C (Vollenweider et al. 2011). At these colder temperatures, infection with the parasite had a greater effect on stored energy reserves as lipid and energy content of herring was at its lowest due to reduced prey consumption during winter months (Vollenweider et al. 2011). In the current study, we found that eggs from *Ichthyophonus*-infected females had increased lipid and water content compared

with eggs from non-infected females. This could indicate that infected females are allocating more resources to their eggs in an attempt to increase the eggs' chances of survival. Chinook salmon, like most teleost fishes, are oviparous and produce yolk containing eggs (Lubzens et al. 2010). In fishes, the major elements of the egg, such as yolk and eggshell, are proteins and lipids, which are allocated from the liver, and taken up from prey resources. Nutrients are then transported to the oocyte for uptake (Arukwe and Goksoyr 2003). Maternal effects on offspring decrease as offspring mature and become zero with increasing age of the offspring (Heath et al. 1999). Egg condition is directly associated with alevin size at hatching; consequently, it is a key factor of survival during important early mortality phases (Kamler 1992, 2005).

While we did find differences in lipid allocation to the eggs of *Ichthyophonus*-infected and non-infected Chinook salmon, which were unexpectedly higher in infected fish, we did not investigate lipid classes in more detail. Yolk contains essential fatty acids, phospholipids, and triacylglycerol that are necessary for development and as an energy resource for successful hatching and survival (Wiegand 1996, Rainuzzo et al. 1997, Bachan et al. 2012). As the egg matures, changes in lipid classes and fatty acid composition occur. For example, phosphatidylcholine is reduced during embryogenesis, but is one of the most important lipids present in early life stages of eggs (Cowey et al. 1985, Fraser et al. 1988, Pickova et al. 1997). Thus, the presence of an overall higher lipid amount in *Ichthyophonus*-infected salmon eggs may not necessarily translate into better quality eggs. Similarly, the presence or absence of vitamins and antioxidants of spawning Chinook salmon can affect the quality of eggs they produce. For example, vitamin A (in the form of retinol and carotenes) is sequestered by the female from muscle and liver and is bound to egg yolk proteins that are essential for egg viability and normal development of embryos (Palace and Werner 2006). Ascorbic acid is also utilized extensively during the alevin stage and must be restored during the first feeding session of the fish to ensure healthy growth (Cowey et al. 1985, Blom and Dabrowski 1995). Thiamine, or vitamin B1, deficiency in adult salmon can cause the occurrence of M74 syndrome in stocked fish in artificial systems, which is lethal in the alevin stage of development (Pickova et al. 1998). Thiamine deficiency, also known as Cayuga syndrome, in the Finger Lakes Atlantic salmon caused 100 % mortality in alevins, which was triggered by an unbalanced diet *prior* to spawning (Fisher et al. 1996, Brown et al. 2005). Further, reduced levels of vitamin E can decrease alevin survival and can increase the incidence of developmental abnormalities (Izquierdo et al. 2001). Although the current study did not find

any evidence of impaired hatching success or growth in alevins from *Ichthyophonus*-infected parents relative to non-infected parents, future studies should focus on how infection with *Ichthyophonus* in wild fish may influence lipid class availability, as well as levels of developmentally important vitamins and antioxidants.

Successful fertilization depends on the quality of sperm produced and can be defined by its ability to successfully fertilize an egg. In the current study, survival of eggs fertilized with sperm from an *Ichthyophonus*-infected male was somewhat lower relative to eggs fertilized with sperm from non-infected males. However, this finding should be viewed with caution due to sample size limitations. Similar to the current study, no difference was detected between sperm cell counts from parasitized and non-infected wild-caught Arctic char *Salvelinus alpinus* (Liljedal et al. 2008). Conversely, sockeye salmon produced less sperm when they were infected with various parasites for more than a year (Konovalov 1995). When milt is released from adults externally for fertilization, water activates sperm motility, which is a large energy sink for spermatozoa as flagellar movement has to be maintained (Bobe and Labbe 2010). We did not see a significant difference in sperm cell counts between *Ichthyophonus*-infected and non-infected males, but it is possible that other factors, such as motility and energetics, are affected by disease. As discussed for eggs, lipid content of the diet also affects milt lipid composition. Thus, even if all essential nutritional obligations are met, antioxidants and certain lipid classes, such as phospholipids and polyunsaturated fatty acids, are crucial to the fertilizing ability and genetic veracity of sperm (Labbe et al. 1995, Bell et al. 1996, Pustowka et al. 2000, Asturiano et al. 2001, Dabrowski and Ciereszko 2001). While the current study did not find any differences in sperm quality parameters (i.e., live/dead cells), we did not investigate many important gamete quality variables, such as sperm cell motility and energetics, antioxidant effects, heat-shock protein expression, gamete DNA damage, and associated potential infertility as a result of disease (e.g., Dabrowski and Ciereszko 2001, Rurangwa et al. 2004, Migaud et al. 2013).

In the current study, there are several caveats to take into consideration. First, there was a limited sample of *Ichthyophonus*-infected salmon for analysis. Yukon River Chinook salmon returns have been below expectations for several years; therefore, in 2011 and 2012, we were not able to obtain independent samples separate from ADF&G. Due to this constraint, we were only able to conduct the fertilization study and hatching success of non-infected and *Ichthyophonus*-infected

eggs in 2010. In 2010, our fertilization study was cut short due to a dewatering event that led to the early demise of all alevins in the study. Also in 2010, one sperm sample from an *Ichthyophonus* positive male was lost in a refrigerator malfunction, where the refrigerator lost power overnight, resulting in milt becoming too warm and all cells dying and clumping.

The Yukon River Chinook salmon population has declined in recent years, and one possible contributor could be the parasite *Ichthyophonus*. In the current study, we examined effects of the disease on spawning Chinook salmon and their offspring. We found no difference in hatching success of eggs from *Ichthyophonus*-infected and non-infected fish, nor did we find a measureable effect of disease on proximate composition parameters of adults and offspring. Therefore, our study suggests that *Ichthyophonus* does not have an effect on offspring survival, and that this parasite may not substantially change the quality of escapement in the Salcha River tributary. However, future studies should examine more fully the potential effects of *Ichthyophonus* on gamete quality, including lipid classes, vitamins, and DNA damage. *Ichthyophonus*-infected Chinook salmon sampled in this study were selected from the successful survivors after a long spawning migration, and the effect of *Ichthyophonus* could be more acute in the proportion of the population that did not make it to the spawning grounds.

2.6 Acknowledgements

We thank ADF&G for their cooperation with this project. This project was supported by the Pollock Conservation Cooperative Research Center through a grant awarded to L. Horstmann-Dehn. Fish resource permits were issued by ADF&G for the sampling years of 2010-2012 as follows; SF2010-129, SF2011-068, and SF2012-071, respectively. This study was authorized by the University of Alaska Fairbanks Institutional Animal Care and Use Committee under permit #170553-3.

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Table 2.1: Adult Chinook salmon morphometrics and egg percent survival Morphometrics and percent survival to hatching (mean±1SD, median, and data range) of adult Chinook salmon sampled during 2010 – 2012 *(n* = sample size). Sample size for egg survival refers to the number of hatching trays and/or crosses in case of *Ichthyophonus*-infected fish. Samples were analyzed using a Kruskal-Wallis non-parametric test with a *p*-value ≤ 0.05 considered significant, H_0: mean ranks of the two populations are equal.

	***Ichthyophonus*-infected**	**Non-infected**	***p*-value**
Total Length [mm]			
Sample Size	*n* = 7	*n* = 88	
Sexes	(4 males, 3 females)	(43 males, 45 females)	
Mean±1SD	895±115	870±115	0.29
Median	865	885	
Range	770-1120	590-1125	
Girth [mm]			
Sample Size	*n* = 7	*n* = 88	
Mean±1SD	423±63	421±72	0.63
Median	409	422	
Range	342-544	253-610	
Survival [%]			
Sample Size	*n* = 8	*n* = 8	
Mean±1SD	24.4±29.8	41.0±24.8	0.25
Median	11.3	50.2	
Range	0.0-76.4	0.0-64.3	

Table 2.2: Water quality parameters for the hatchery system. Water quality parameters for the University of Alaska Fairbanks hatchery system. Each parameter was measured 46 times (*n*) over the course of approximately 3 months (July-September 2010). Hardness was consistently greater than 342.0 mg/L, which is the maximum concentration measurable by the water quality kit we used, therefore the data range was not recorded (NA).

Water Quality Parameter	Mean ± 1SD	Data Range
Temperature [°C]	10.6±0.8	10.1-13.7
pH	7.5±0.2	7.0-8.0
Dissolved Oxygen [%]	101.4±1.5	98.0-104.8
Salinity	3.5±0.5	2.5-4.5
Total ammonia [mg/L]	1.8±0.5	0.4-3.5
Nitrite [mg/L]	0.01±0.01	0.0-0.004
Alkalinity [mg/L]	70.7±17.3	51.3-102.6
Hardness [mg/L]	>342.0	NA

Table 2.3: Proximate composition of adult Chinook salmon muscle. Proximate composition (dry weight) of adult Chinook salmon muscle, 2010 – 2012 *(n* = sample size). Kruskal-Wallis non-parametric test was used with a *p*-value ≤ 0.05 considered significant, H_0: mean ranks of the two populations are equal.

	***Ichthyophonus*-infected** $n = 7$ (4 males, 3 females)	**Non-infected** $n = 85$ (43 males, 42 females)	***p*-value**
%Water			
Mean±1SD	79.7±3.0	78.1±3.5	0.14
Median	80.0	78.2	
Range	75.3-85.0	70.6-85.5	
%Crude Protein			
Mean±1SD	16.7±3.9	19.6±3.8	0.07
Median	15.5	18.7	
Range	11.6-23.2	13.3-26.4	
%Lipid			
Mean±1SD	21.5±7.6	19.4±5.7	0.82
Median	18.8	18.5	
Range	12.3-31.1	10.2-36.4	
Energy Content [kJ/g]			
Mean±1SD	5.6±0.5	5.6±0.6	0.11
Median	5.4	5.4	
Range	5.2-6.5	4.7-7.6	

Table 2.4: Proximate composition of Chinook salmon eggs. Proximate composition (dry weight) of Chinook salmon eggs sampled during 2010 – 2012 *(n* = sample size). Egg crude protein and energy content were non-normally distributed, thus, a Kruskal-Wallis test was used with a *p*-value ≤ 0.05 considered significant, H_0: mean ranks of the two populations are equal. Egg lipid and water content were normally distributed with equal variance, therefore, a one-way ANOVA was used with a *p*-value ≤ 0.05 considered significant, H_0: means of the two populations are equal.

	***Ichthyophonus*-infected** $n = 3$	**Non-infected** $n = 42$	***p*-value**
%Water			
Mean±1SD	66.5±3.4	62.0±2.4	0.005
Median	66.5	61.9	
Range	63.1-69.8	57.7-67.7	
%Crude Protein			
Mean±1SD	37.8±0.06	36.4±2.9	0.41
Median	37.8	37.8	
Range	37.8-37.9	30.2-40.0	
%Lipid			
Mean±1SD	30.2±2.2	22.8±5.1	0.02
Median	29.1	22.4	
Range	28.8-32.7	12.9-34.1	
Energy Content [kJ/g]			
Mean±1SD	6.0±0.1	6.4±0.5	0.05
Median	6.0	6.2	
Range	6.0-6.1	5.9-7.9	

Table 2.5: Proximate composition of Chinook salmon alevin. Proximate composition (dry weight) of Chinook salmon alevins 2010 – 2012, *(n* = sample size) from either *Ichthyophonus*-infected or non-infected parents. One-way ANOVA was used with a *p*-value ≤ 0.05 considered significant, H_0: means of the two populations are equal.

	***Ichthyophonus*-infected** *n* = 5	**Non-infected** *n* = 30	***p*-value**
%Water			
Mean±1SD	73.5±0.9	74.4±2.3	0.08
Median	74.0	72.9	
Range	73.4-75.6	70.8-80.9	
%Crude Protein			
Mean±1SD	14.5±1.8	14.4±1.3	0.88
Median	14.7	14.5	
Range	11.6-16.6	11.2-16.9	
%Lipid			
Mean±1SD	29.4±2.7	28.5±3.4	0.58
Median	29.4	28.7	
Range	25.9-33.2	21.9-34.1	
Energy Content [kJ/g]			
Mean±1SD	6.0±0.1	5.8±0.4	0.28
Median	6.0	5.9	
Range	5.8-6.1	5.0-6.8	

Table 2.6: Chinook salmon alevin morphometrics. Morphometrics (i.e., length, weight, yolk sac height) of Chinook salmon alevins hatched in 2010 from either *Ichthyophonus*-infected or non-infected parents. One-way ANOVA was used with a p-value ≤ 0.05 considered significant, H_0: means of the two populations are equal.

	***Ichthyophonus*-infected** $n = 5$	**Non-infected** $n = 8$	***p*-value**
Length [mm]			
Mean±1SD	24±2	25±2	0.40
Median	24.8	24.6	
Range	21-26	20-27	
Weight [g]			
Mean±1SD	0.2524±0.0297	0.2661±0.0382	0.69
Median	0.2358	0.2661	
Range	0.2251-0.2949	0.1986-0.3088	
Yolk Sac Height [mm]			
Mean±1SD	7±0	7±0	1.00
Median	7	7	
Range	NA	6-8	

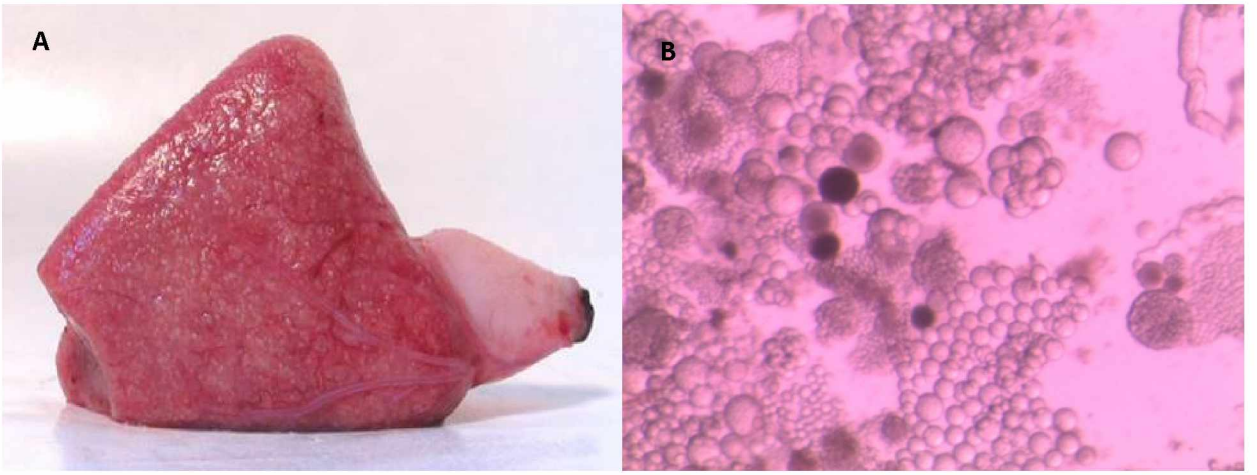

Figure 2.1: Clinical signs of *Ichthyophonus* in Chinook salmon. Clinical signs of *Ichthyophonus* infection in the heart of a Chinook salmon (A); white diffuse lesions are visible. (B) *Ichthyophonus* spores at 50x magnification. Photos by L. Horstmann-Dehn (A) and C. Whipps (B).

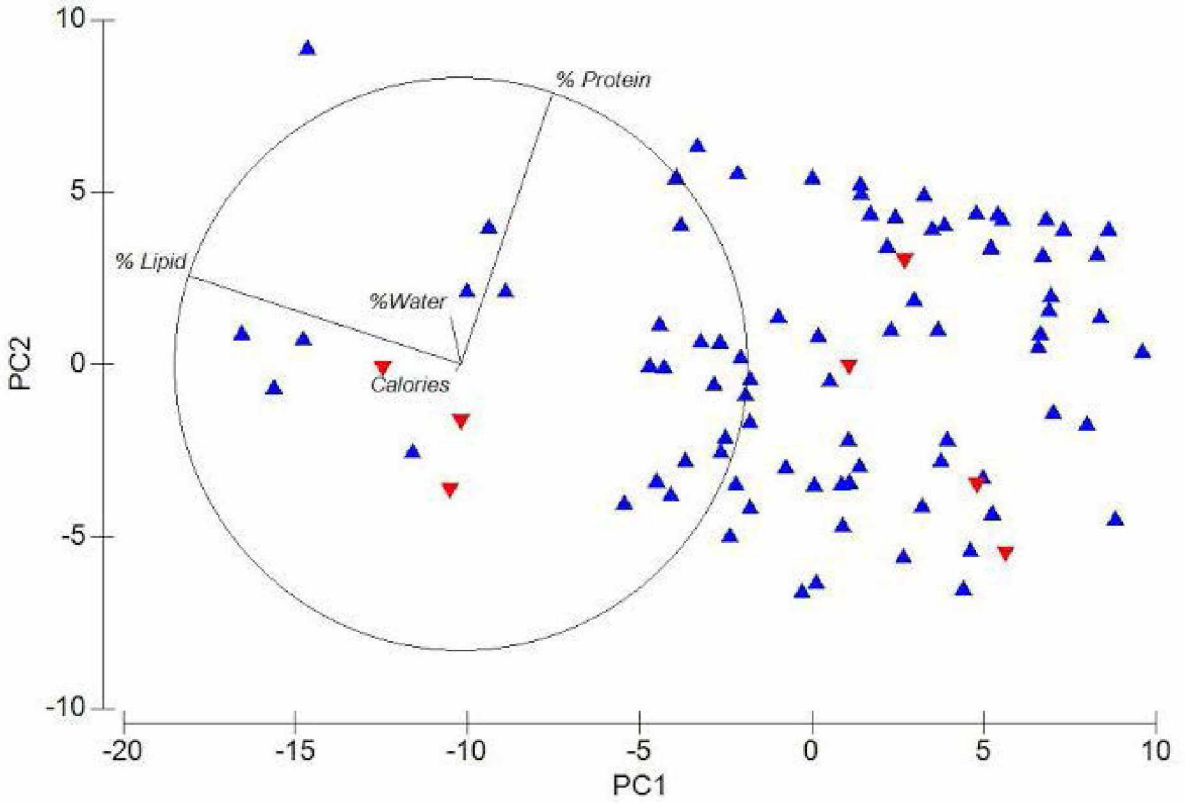

Figure 2.2: Principal components analysis of spawning Chinook salmon proximate composition. Principal components analysis (PCA) of proximate composition parameters (% water, % lipid, % crude protein, and energy content) of spawning Chinook salmon muscle, 2010 – 2012. Red triangles represent *Ichthyophonus*-infected Chinook salmon and blue triangles show non-infected fish. PC1 explains 60.4 % of variability and PC2 accounts for 21.0 % for a total of 81.3 % variability explained. Vectors for the variables are shown (black lines), and vector length indicates the importance of the variable contribution.

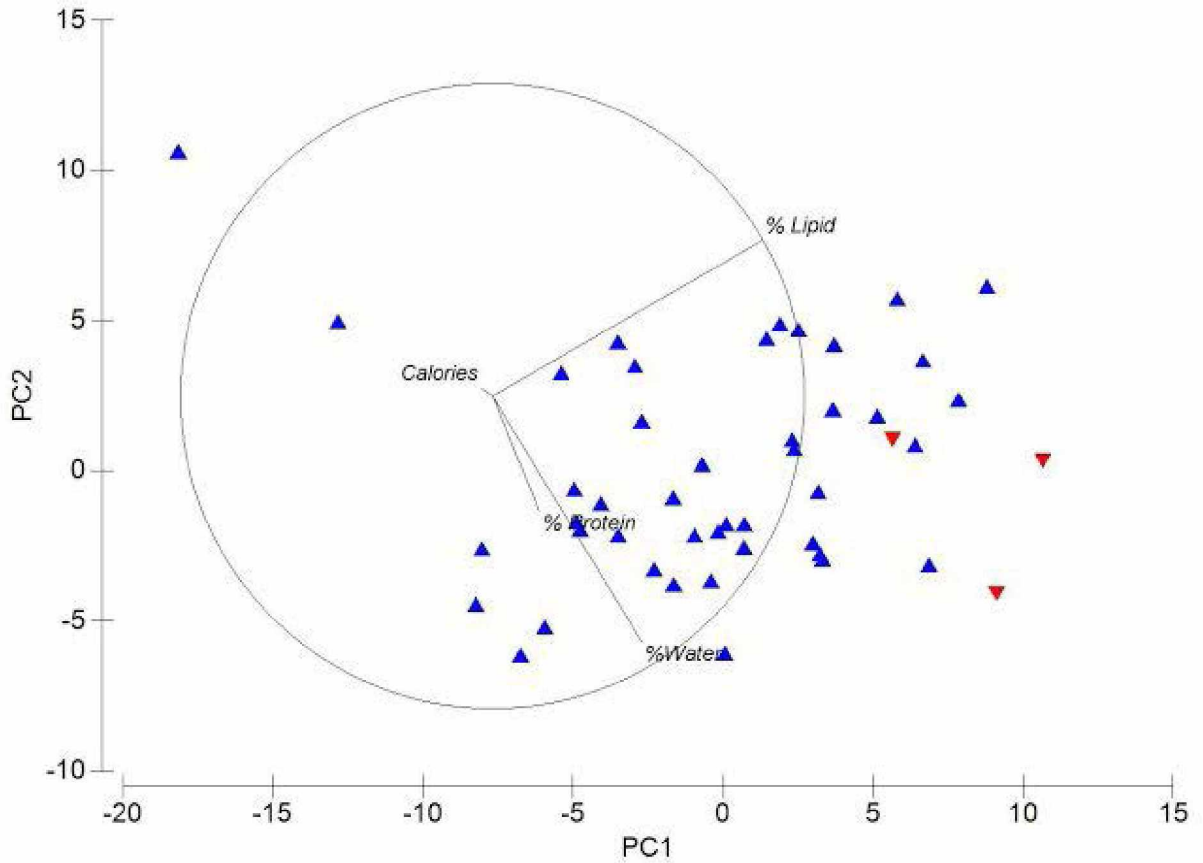

Figure 2.3: Principal components analysis of egg proximate composition. Principal components analysis (PCA) of proximate composition parameters (% water, % lipid, % crude protein, and energy content) of Chinook salmon eggs, 2010 – 2012. Red triangles represent eggs from *Ichthyophonus*-infected adults and blue triangles show eggs from non-infected adults. PC1 explains 62.2 % of variability and PC2 accounts for 25.0 % for a total of 87.3 % variability explained. Vectors for the variables are shown (black lines), and vector length indicates the importance of the variable contribution.

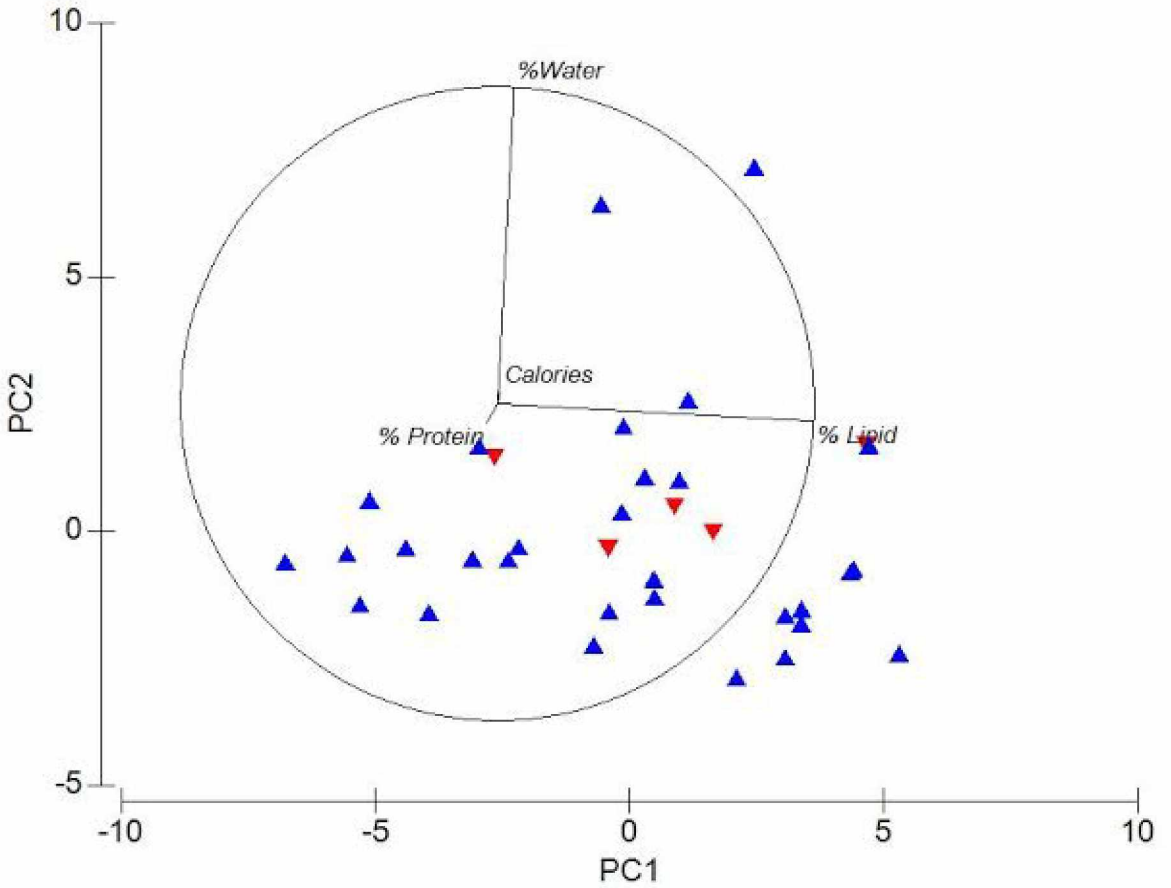

Figure 2.4: Principle components analysis of Chinook salmon alevin proximate composition. Principal components analysis (PCA) of proximate composition parameters (% water, % lipid, % crude protein, and energy content) of Chinook salmon alevins, 2010 – 2012 . Red triangles represent alevins from *Ichthyophonus*-infected adults and blue triangles show alevins from non-infected adults. PC1 explains 62.3 % of variability and PC2 accounts for 27.5 % for a total of 89.8 % variability explained. Vectors for the variables are shown (black lines), and vector length indicates the importance of the variable contribution

Chapter 3: Effect of *Ichthyophonus* on blood plasma chemistry and cortisol concentrations of spawning Yukon River Chinook salmon and their offspring[2]

3.1 Abstract

Ichthyophonus hoferi is a parasite in Alaska Chinook salmon *Oncorhynchus tshawytscha*. In this study, we determined if spawning Chinook salmon in the Yukon River drainage, and their resulting alevins, exhibited a measurable stress response (i.e., elevated plasma cortisol concentrations) and detectable changes in selected blood plasma chemistry parameters when infected with *Ichthyophonus*. In 2010, 2011, and 2012, spawning adult Chinook salmon were collected from the Salcha River, Alaska, and the prevalence of *Ichthyophonus* in these fish was 7.8 %, 6.3 %, and 8.3 %, respectively. Presumably healthy and *Ichthyophonus*-positive parents were cross-fertilized to investigate potential second-generation effects as a result of *Ichthyophonus* infection. We found no significant difference in cortisol concentrations in blood plasma between *Ichthyophonus*-positive and negative adults or between alevins from *Ichthyophonus*-positive and negative parents. There were no significant differences in blood plasma parameters (e.g., alanine aminotransferase, creatine kinase, glucose) of *Ichthyophonus*-negative and positive adults, with the exception of aspartate aminotransferase, which was significantly higher in plasma of *Ichthyophonus*-negative adults. All clinical chemistry parameters for alevins from both *Ichthyophonus*-negative and positive parents were not significantly different. Overall, *Ichthyophonus* does not appear to affect spawning Chinook salmon or offspring blood chemistry, as there were no measurable effects on the second generation as a result of *Ichthyophonus*. Based on this study, which has a limited sample size and low prevalence of *Ichthyophonus,* offspring of Salcha River Chinook salmon appear to suffer no disadvantage as a result of *Ichthyophonus* infection in their parents.

Key Words: *Ichthyophonus*, Chinook salmon, Yukon River, Salcha River, cortisol concentration, clinical blood chemistry

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[2] Floyd-Rump TP, Horstmann-Dehn LA, Atkinson S, Skaugstad C. Effect of *Ichthyophonus* on blood plasma chemistry and cortisol concentrations of spawning Yukon River Chinook salmon and their offspring. Prepared for submission to Diseases of Aquatic Organisms

3.2 Introduction

Chinook salmon *Oncorhynchus tshawytscha* are a fundamental source of sustenance, economics, and culture in Alaska and the Pacific Northwest, providing food for marine mammals, predatory fishes, seabirds, terrestrial animals (including humans), and providing marine-derived nutrients to river banks after spawning (Cederholm et al. 1999). Chinook salmon are anadromous and semelparous, and fish return from the ocean to rivers, such as the Yukon River in Alaska from May to August to spawn (Healey 1991). Chinook salmon in the Yukon River drainage follow the stream-type life history behavior (Healey 1991); once hatched, most Chinook salmon migrate to the ocean about one year after hatching, with some delaying migration for an additional year (Behnke 2002). Chinook salmon spawning in the upper reaches of the Yukon River drainage undertake one of the longest migrations (between 2400 – 3200 km) known for any salmonid in the world (Behnke 2002).

Salmonids in the Yukon River drainage are an essential part of Alaska's fishery, and Chinook salmon are particularly valued, because of their high lipid content (Healey 1991). More than 30 Yukon River communities and 2000 households depend on salmon, including Chinook salmon, as part of their subsistence way of life (Merritt 2001, Jallen and Hamazaki 2011, Carroll and Hamazaki 2012). Chinook salmon returns in 2007, 2008, and 2009 were lower than expected based on reported spawning escapements in the brood years (JTC 2008, 2009, 2011). In response, a Chinook salmon directed commercial fishery on the Yukon River mainstem did not occur in 2008 and 2009 for the first time in almost a decade (JTC 2011), and the subsistence fishery was reduced by up to 50 %. Even with these fishery restrictions, the interim management escapement goal into Canada as part of the U.S. Pacific Salmon Treaty obligations in 2008 was not met. The management plan in 2009 included the protection of the first pulse of Chinook salmon entering the Yukon River and closure of all fisheries (JTC 2011). Chinook salmon stocks in the Arctic-Yukon-Kuskokwim region (AYK) have continued to have low abundance, and salmon returns have not met pre-season expectations based on escapement in the corresponding brood years (JTC 2011, 2013, 2014). Consequently, the subsistence fishery was reduced up to 50 % and, in 2014, all Chinook salmon harvest, including subsistence, was closed on the Yukon River. In 2015, the run was still below the historical average, but escapement into Canada was met and some subsistence fishing was allowed (ADF&G 2015). While the ultimate cause(s) for

the continued run failures of Chinook salmon remain unknown, infectious diseases play a role in some other salmon population declines (Kocan et al. 2004, Kocan and Hershberger 2006). Disease may be a contributing factor, either due to pathogen-induced mortality, reduced fecundity, or the inability of Chinook salmon to successfully migrate and spawn.

Ichthyophonus hoferi (*Ichthyophonus* hereafter) is a marine-derived protozoan parasite infecting a variety of marine and anadromous fish species, including salmonids (McVicar 1999, Kocan et al. 2004, Tierney and Farrell 2004, Gavryuseva 2007). This parasite was identified by the Alaska Department of Fish and Game (ADF&G) and the U.S. Fish and Wildlife Service in the mid-1980s after fishers reported white pustules on the heart, liver, and musculature of Chinook salmon returning to spawning tributaries in Alaska (Kocan et al. 2004). By the early 2000s, *Ichthyophonus* prevalence had reached 25-30 % in Chinook salmon in the mainstem of the Yukon River (Kocan et al. 2003, 2004), and appears to be cyclical over time (Zuray et al. 2012). As Chinook salmon abundance fluctuates, prevalence of the disease also changes (Kocan et al. 2004, Zuray et al. 2012), as is typical of a parasite cycle where increased numbers of individuals in an area can increase the incidence of disease and *vice versa* (Altizer et al. 2006). Evidence suggests that *Ichthyophonus* may cause pre-spawning mortality in Chinook salmon (Kocan et al. 2004, 2006, 2009). Since 2003, the prevalence of *Ichthyophonus* in Chinook salmon has declined continuously and appears to be correlated with a greater than 50 % decline in the population abundance of Chinook salmon in the Yukon River (Zuray et al. 2012). Effects of the parasite on the host include a higher total body lipid and protein loss relative to healthy fish by approximately 30 % (Vollenweider et al. 2011). In *Ichthyophonus*-positive sockeye salmon *Oncorhynchus nerka*, oxygen uptake and exercise capabilities are reduced (Tierney and Farrell 2004), and fish appear to compensate by increasing heart mass and cardiac output (Kocan and Hershberger 2006). Consequently, lower stamina and swim speed have are also associated with *Ichthyophonus* infection (Kocan et al. 2009). Due to these effects, *Ichthyophonus* prevalence can be higher in lower parts of the Yukon River compared with the spawning grounds as fish die before reaching their final destination (Kocan and Hershberger 2006).

Environmental stressors are well recognized for their impact on fish health (Marcogliese 2004), and disease caused by *Ichthyophonus* may be exacerbating morbidity of salmon in Alaska (Hamazaki et al. 2013). Temperature changes influence disease processes in poikilotherms (Finn

and Nielson 1971). For example, adult rainbow trout *Oncorhynchus mykiss* infected with *Ichthyophonus* experienced 100 % mortality at water temperatures ranging from 15 to 20 °C, but a higher proportion of infected fish survived when temperatures were less than 15 °C (Okamoto et al. 1987). Similarly, Kocan et al. (2009) reported higher parasite load and faster die-offs with increased temperature, as well as reduced swimming performance in *Ichthyophonus*-positive rainbow trout. In-river conditions in the Yukon River have changed over the past 30 years, with June water temperatures having increased by approximately 2.5 °C (Kocan et al. 2004, Kahler et al. 2007). Fish are particularly sensitive to higher temperatures because it changes the rate of physiological processes, disrupts the structure of macromolecules, and hastens the growth of pathogens (Fry 1971, Marcogliese 2001, Crockett and Londraville 2006). Increased water temperatures affect all life stages of fish, but the larval stage is most sensitive to environmental fluctuations as temperature affects size at hatching, developmental rate, larval duration, and survival (Pankhurst and Munday 2011).

Environmental or induced stressors may disturb the normal physiological equilibrium of teleost fishes by driving a repartitioning of energy in the body (Mommsen and Moon 2001), thus compromising survival and fitness (Schreck et al. 2001). Stress lowers the ability of fish to maintain homeostasis and carry out actions crucial for endurance, growth, and reproduction (Schreck 1982). The most common glucocorticoid hormone found in salmonids is cortisol (Donaldson 1981, Passino 1984), which is often utilized as a gauge of stress (Fevolden et al. 1993) and serves as a reliable bioindicator of stress in fishes. For example, cortisol concentrations in red drum *Sciaenops ocellatus* increased with extended periods of transportation stress (Thomas and Robertson 1991). Acute and chronic stress can induce a variety of physiological responses in organisms, many of which are negative (Schreck 1982). In general, increased cortisol levels are associated with increased disease susceptibility (Fevolden et al. 1993). Brown trout *Salmo trutta* with prolonged increased plasma cortisol concentrations exhibited increased sensitivity to bacterial and fungal diseases and increased mortality (Pickering and Pottinger 1989). Prolonged elevated cortisol concentrations can also affect a wide range of reproductive parameters, such as gonad size and testosterone concentrations (Carragher et al. 1989). Stressed fish generally have lower stamina, which in turn can result in reduced reproductive output (Carragher et al. 1989, Kocan and Hershberger 2006) and fecundity (Hall et al. 2011). Similarly, vitellogenesis is adversely influenced by high concentrations of

corticosteroids (Pankhurst and Van Der Kraak 2000, King et al. 2003). In teleost fishes, hormones, such as cortisol, are discharged into the yolk sac during oogenesis in amounts that reflect female plasma concentrations (Eriksen et al. 2013), suggesting that maternal endocrine condition at the time of spawning may affect offspring.

Measurement of blood plasma parameters can be useful indicators of tissue damage due to disease or other stressors (Grizzle and Kiryu 1993, McPherson and Pincus 2011), and monitoring the health of free-ranging animal populations does not necessarily require lethal procedures when utilizing blood. Enzyme activities, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) can offer an understanding of the physiological response of an animal to disease and stress (Schreck et al. 1997, Yousaf and Powell 2012). Diseases, such as *Ichthyophonus*, cause heart and skeletal muscle inflammation, which has been positively correlated with increased levels of AST and CK (Marty et al. 1998, Yousaf and Powell 2012). Further, intracellular enzymes, such as ALT, AST, and CK, are released by cell damage or cell death (Wagner and Congleton 2004, McPherson and Pincus 2011). The involvement of these enzymes in acute and chronic disease processes, in particular with regard to tissue damage, makes these blood plasma parameters good potential bioindicators for the necrotic and inflammatory action associated with *Ichthyophonus* infection. Elevated level of blood glucose (GLU) is another indicator of stress in fishes (Van Waarde et al. 1990, Wendelaar Bonga 1997). Blood GLU levels in Atlantic salmon *Salmo salar* and rainbow trout increased in conjunction with increased stress (Fevolden et al. 1991). Another potential bioindicator for homeostatic disruption is alkaline phosphatase (ALP). While the exact function of ALP is not known, it is thought to act on the movement of ions and absorption of water across cell membranes (Gasser and Kirschner 1987, Moss 1992). Atlantic salmon infected with salmon lice *Lepeophtheirus salmonis* had elevated concentrations of ALP (as well as cortisol) relative to non-infected fish (Ross et al. 2000). Alkaline phosphatase (ALP) is also responsive to nutritional changes, such as food intake and body condition (Wagner and Congleton 2004), with fasted fish having 50-75 % less ALP activity compared with fed fish (Sauer and Haider 1979, Bucher 1990, Congleton and Wagner 2006). Monitoring overall fish health needs to utilize multiple blood parameters, because fish can exhibit considerable variation in their physiological responses to the same stressor (Barton 2000).

The purpose of this study was to determine if Chinook salmon were exhibiting a measurable stress and physiological response to infection with *Ichthyophonus*. We hypothesized that spawning Chinook salmon infected with *Ichthyophonus* would exhibit a stress response resulting in higher concentrations of cortisol and GLU compared with *Ichthyophonus*-negative salmon, as well as measureable increases in tissue damage indicators (e.g., ALT, AST, and CK). We further hypothesized that cortisol concentrations and some blood plasma parameters, such as CK and AST, propagate to the second generation, such that offspring produced by *Ichthyophonus*-positive parents would differ from those of *Ichthyophonus*-negative parents.

3.3 Methods

3.3.1 Sample Collection

In 2010 and 2011, Chinook salmon were sampled via electrofishing from the Salcha River, Alaska (approximately River km 39 at the following coordinates: N64.472, W146.971). In summer 2010, we collected 51 Chinook salmon (27 females [including 3 freshly dead] and 24 males) from July 29th to August 1st. On July 19th 2011, we sampled 32 Chinook salmon (15 females and 17 males). On July 26th 2012, we sampled 12 Chinook salmon (6 females and 6 males) at the Salcha River boat ramp (N64.464, W146.967); these fish were captured using rod and reel sport fishing gear. The lower sample size and different capture gear used in 2012 was due to permit restrictions as a result of low Chinook salmon returns to the Yukon and Salcha rivers.

After capture, adult Chinook salmon were held in net pens (1.2 m x 1.2 m x 2.4 m, with a 3.8-cm mesh) for 1 to 2 days, and were maintained in the river current for continuous water flow. Fish were held at a maximum density of 12 fish per pen, with males and females kept separated. The ripeness of females was characterized by loss of skein structure, and was determined by gentle palpation of the belly and deposition of eggs through the ovipositor. Milt was readily available from males upon capture; therefore, female ripeness determined when fertilization could commence. Fish were euthanized by cranial concussion, followed by exsanguination. Following this, blood was collected from the caudal vein after the peduncle was cut. Whole blood was collected in sterile BD Vacutainers® coated with sodium heparin and centrifuged at 1900 g for 10 minutes (VWR® Clinical 50 Centrifuge). Next, plasma was pipetted into cryovials and

immediately flash frozen in liquid nitrogen. Once all samples were collected and the site was thoroughly cleaned for the day, samples were transported to the University of Alaska Fairbanks and stored at -80 °C until analysis.

To avoid cross-contamination with *Ichthyophonus* across fish and samples, tissues were collected using extreme care with sterile, disposable sampling supplies. The ventral surface of each fish was cleaned of mucus and blood and cut with a sterile blade to expose the heart. All fish were examined internally for typical clinical signs of *Ichthyophonus* infection, which are white pustules on the heart, liver, or kidney (Figure 3.1a). A piece of cardiac muscle (~1 g) was removed with a second sterile blade and cultured in MEM-5 supplemented with 5 % fetal bovine serum, penicillin, streptomycin, and gentamycin (Kocan and Hershberger 2006). The tissue was then incubated at 14 °C and examined daily for *Ichthyophonus* spores (Figure 3.1b) to confirm clinical infection with *Ichthyophonus* at the State University of New York, Syracuse, NY (Whipps et al. 2005). A second sub-sample of cardiac muscle was placed in 95 % ethanol and shipped to Purdue University, West Lafayette, IN, for molecular confirmation of infection. The presence of *Ichthyophonus* 18S rDNA was evaluated using polymerase chain reaction (PCR) following the procedure described by Whipps et al. (2005).

3.3.2 Analysis of Plasma Chemistry and Alevin Homogenates

Spawning Chinook salmon blood plasma was analyzed with an Abaxis VetScan using the comprehensive diagnostic profile reagent rotor and the avian/reptilian profile plus rotor. The following diagnostic parameters were analyzed (usually within 8 hours of collection) in Chinook salmon plasma samples: albumin (ALB), ALP, ALT, amylase (AMY), AST, bile acids (BA), blood urea nitrogen (BUN), calcium (Ca), CK, cortisol, creatinine (CRE), globulin (GLOB), GLU, potassium (K), sodium (Na), phosphorous (P), uric acid (UA), total bilirubin (TB), and total protein (TP). For alevins, the diagnostic parameters listed above were determined on total body homogenates after Franson (1982). In 2010, alevins were obtained from this study's fertilization trial (Chapter 2). In 2011 and 2012, the Alaska Department of Fish and Game (ADF&G) Division of Sport Fish in Fairbanks collected spawning Chinook salmon from the Salcha River for rearing offspring in the state hatchery, and the resulting alevins were acquired for analysis in this study; parentage (i.e., *Ichthyophonus*-positive or *Ichthyophonus*-negative was known for all alevins). Approximately 1 g of tissue (or ~5 alevins) was placed in a glass homogenization tube,

and then 9 mL of cold phosphate buffer (0.1 M, pH 7.4) was added to the tube for homogenization (Franson 1982). Using a glass rod, the sample was homogenized for 2-3 minutes, or until all large particulates were dissolved. Next, the sample was centrifuged for 20 minutes at 1000 g, and supernatants were removed using a serological pipette. Samples were refrigerated at 4 °C immediately after preparation and were analyzed on an Abaxis VetScan Classic, using a comprehensive diagnostic profile reagent rotor and an avian/reptilian profile plus rotor, within 4 hours of homogenization.

3.3.3 Cortisol Radioimmunoassay

Cortisol concentrations were determined using a solid phase single antibody radioimmunoassay (RIA) (Siemens Coat-A-Count® Kit). Plain 12x75 mm polypropylene tubes were used in duplicate for all samples and standards. Cortisol calibrators containing 0, 5, 10, 50, 100, 200, and 460 ng of cortisol per mL (ng/mL) in processed human serum was used to create a standard curve. Before Chinook salmon plasma samples were tested, the assay was validated with tests for parallelism (Figure 3.2a) and accuracy (Figure 3.2b). For each sample, 25 µL of each standard was added to each tube in duplicate, with the zero calibrator added to the nonspecific binding tube. Then 1 mL of ^{125}I cortisol was added to every tube, and all tubes were incubated in a water bath for 45 minutes at 37 °C. Sample reactivity was determined with a gamma counter. Any samples that had a 10 % or greater difference in counts between duplicates or counts outside the range of the standard curve were diluted and reanalyzed.

3.3.4 Cortisol Extraction and Assays of Alevin Homogenates

Cortisol concentrations were assessed in total body-homogenized alevins using the solid phase single antibody RIA as described above. One individual alevin, weighing approximately 0.2 g, was placed in a 2 mL cryovial with 1.7-mm ceramic homogenizing beads, and then 1 mL of 200-proof ethanol was added to this cryovial. All samples were homogenized for 45 seconds using a Disruptor Genie® at a speed of 6.5 m/sec. The samples were then iced, and these steps were repeated a total of eight times to ensure proper homogenization. Samples were centrifuged for 10 minutes at 3000 g (Beckman GS-6R), and then 0.5 mL of the supernatant was aspirated into 13x100 mm disposable glass culture tubes and evaporated under compressed nitrogen gas, while incubating in a 25 °C water bath. The supernatant residue was purified in a series of extractions

using ethanol:acetone (4:1 v/v), diethyl ether, acetonitrile, and hexane (Keller et al. 2009). After the final extraction, hexane was added, and the bottom layer was aspirated and then evaporated under nitrogen. The final sample was stored at -80 °C.

Before analysis, the sample homogenates were reconstituted in 500 µL of phosphate-buffered saline (pH 7.5) with 1 % bovine serum albumin. Samples were sonicated using a tissue tearor (BSP 780CL-07) for 3 minutes at the highest setting. Cortisol concentrations were then measured as described above. Because some samples contained low cortisol concentrations, samples were rerun at a higher sample volume. Validation (i.e., parallelism and accuracy) for cortisol assays on alevins are presented in Figure 3.3a and 3.3b.

3.3.5 Statistical Analyses

Standard descriptive statistics were compiled, i.e., mean, median, standard deviation (±1SD), and range. The statistical program package R version 3.0.2 as used for all statistical tests, with an alpha of 0.05 considered significant (R Development Core Team 2008). AShapiro-Wilk's normality test and Bartlett's test for equal variances were performed to assess if data met assumptions for ANOVA (analysis of variance). Data were not normally distributed with unequal variances; therefore, a non-parametric Kruskal-Wallis test was used to assess differences between *Ichthyophonus*-positive and negative spawning adults, male and female spawning adults (*Ichthyophonus*-negative only), and their resulting offspring. Principle components analysis (PCA) was performed on all clinical chemistry parameters for adults and alevins using PRIMER-E (Version 6.1.16) to assess the combined effects of all variables when comparing *Ichthyophonus*-negative and *Ichthyophonus*-positive fish and their offspring. In addition, multidimensional scaling on a Bray-Curtis similarity matrix without data transformation was performed for adult Chinook salmon plasma and alevin clinical chemistry parameters using PRIMER-E (stress of 0.10 is fair and >0.2 is poor; Kruskal 1964). For Chinook salmon alevin the PCA plot was generated using normalized Euclidean distance, these data were normalized to depress the impact of CK, as this variable had concentrations several orders of magnitude higher than others.

3.4 Results

In 2010, we collected 51 Chinook salmon (27 females [3 dead], 24 males), and 4 fish were *Ichthyophonus*-positive (3 females, 1 male; 7.8 % prevalence). Note that 1 male and 3 female blood plasma samples were hemolyzed and therefore were unusable for blood chemistry analysis. In 2011, 15 females and 17 males were sampled, and 2 males were infected with *Ichthyophonus* (6.3 %). In 2012, we sampled 12 salmon (6 males, 6 females), and only 1 male was positive for *Ichthyophonus* (8.3 %). Infection with *Ichthyophonus* was confirmed by culture and PCR analysis, and there were no false positives or false negatives. However, one individual sampled in 2010 displayed clinical signs of *Ichthyophonus*, but was not positive by either culture or PCR. Plasma chemistry parameters and cortisol concentrations for spawning Chinook salmon are given in Table 3.1. Variables were not significantly ($p>0.05$) different between *Ichthyophonus*-positive and *Ichthyophonus*-negative spawning Chinook salmon (Table 3.1), with the exception of AST, which was significantly lower in *Ichthyophonus*-positive adults ($p=0.03$). Creatine kinase (CK) ranged from 0.0±0.0 U/L for *Ichthyophonus*-positive adults to 1041.1±2678.2 U/L for *Ichthyophonus*-negative adults, but was not significantly different ($p=0.27$) due to large standard deviations. Principal components analysis (PCA) supports the overall lack of difference between *Ichthyophonus*-positive and *Ichthyophonus*-negative adult Chinook salmon (Figure 3.4 a and b). The first principal component (PC) using blood parameters of spawning Chinook salmon explained 88.0% of the variability, and the second PC explained 10.2 % (Figure 3.4a). However, PCA showed two groups of salmon that separated due to high concentrations of CK, but no clear pattern emerged (Figure 3.4a) after this analysis. When CK was removed from the PCA (Figure 3.4b), two groups were still noticeable, and separation appeared to be mostly due to sex, although not in a uniform manner (Figure 3.4b). The separation was driven by a positive loading of AST and PHOS in PC1, driving the vector values higher, and a negative loading of cortisol in PC1, driving the vector values lower (Figure 3.4b; PC1 explains 84.5 % of the variation, and PC2 explained 13.0 %).

All plasma chemistry parameters for *Ichthyophonus*-negative spawning Chinook salmon were analyzed between males and females (Table 3.2). Variables were not significantly different ($p>0.05$) between male and female salmon, with the exception of ALB ($p=0.0004$), ALP

(p=0.0003), and cortisol (p=0.01), all of which were significantly higher in females compared with males.

All clinical chemistry parameters as well as cortisol for Chinook salmon alevins sired by *Ichthyophonus*-positive and negative adults were not significantly different (p>0.05); concentrations of all parameters are summarized in Table 3.3. The PCA for alevins did show structure but that structure was not explained by either *Ichthyophonus* status nor sex; therefore it did not show any difference between offspring from *Ichthyophonus*-positive and negative parents. PC1 explained 22.4 % of variability, PC2 explained 36.1 % for a total of 58.5 % (Figure 3.5). Multidimensional scaling on a Bray-Curtis similarity matrix without data transformation on clinical chemistry parameters for adults and alevins also showed no dissimilarities between *Ichthyophonus*-infected or non-infected individuals or offspring (stress=0.09 and 0.01 for adults and alevins, respectively).

3.5 Discussion

Analyzing blood plasma parameters, such as cortisol, GLU, and AST, are simple techniques that can provide important information about the physiological status of an animal (Chen et al. 2004). In this study, we investigated the effect of infection with *Ichthyophonus* on spawning Chinook salmon plasma chemistry and potential second generation effects. We did not find any differences in basic clinical chemistry parameters of spawning adults on their terminal spawning grounds as a function of infection with *Ichthyophonus*, with the exception of AST, which was substantially higher in *Ichthyophonus*-negative salmon. Similarly, no differences were observed in clinical chemistry parameters of the resulting offspring.

Semelparous organisms, such as Chinook salmon, reproduce once in their lifetime followed by rapid degenerative physiological changes resulting in death (Healey 1991, Hruska et al. 2010). During their spawning migration, Chinook salmon do not consume prey and endure prolonged endurance exercise for the entire extent of their spawning run (Healey 1991, Morash et al. 2013). Cortisol has been identified as one of the principal steroid hormones in spawning salmonids (Idler et al. 1964), and corticosteroids play a vital role in the catabolism of protein for delivery of metabolic energy in salmonids (Freeman and Idler 1973). Female salmonids generally have higher levels of cortisol than males (Idler et al. 1959, Folmar 1993, Berg et al. 2004), and this was

observed in the current study as well (Table 3.2). Higher cortisol levels in female Chinook salmon could be due to increased estrogen experienced during spawning which can trigger the salmon hypothalamic-pituitary-interrenal axis, whereas in males exogenous androgens do not have this effect (Carruth et al. 2000). Cortisol has also been identified as an important bioindicator for disease (Pickering et al. 1982, Pickering and Pottinger 1989). Chronic increased levels of cortisol increase susceptibility to disease, decrease immune response, and increase wound healing time (Pickering and Pottinger 1989, Godbout and Glaser 2006). Cortisol reduces inflammation in the body, and will increase in blood with infection or disease (Barnes and Adcock 2009). Chinook salmon infected with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, had significantly higher blood plasma cortisol levels than healthy fish (Mesa et al. 1999). Somewhat unexpectedly, the current study did not find a significant difference in blood plasma cortisol concentrations between *Ichthyophonus*-negative and *Ichthyophonus*-positive spawning Chinook salmon. This is likely due to adrenal exhaustion, where the adrenal gland increases cortisol level at the time of river entry and the chronic output of cortisol exhausts the adrenal gland (McConnachie et al. 2012). However, cortisol levels in the current study were higher than reported for pink salmon *Oncorhynchus gorbuscha* (McConnachie et al. 2012, Table 3.1). These higher concentrations in Chinook salmon from the Yukon River drainage relative to other salmonids may be due to longer migration time and distance to reach the final spawning grounds. Chinook salmon cortisol is high at the beginning of migration, and prolonged chronic high cortisol levels could lead to adrenal exhaustion, resulting in low cortisol levels at the end of their spawning migration (McConnachie et al. 2012). Adrenal failure may occur due to the initial increase in number of adrenal cells due to gonadal development followed by marked hyperplasia in the adrenal gland, followed by rapid degeneration (Robertson and Wexler 1959).

Another common indicator of stress in fishes is GLU, primarily due to the direct and indirect actions of cortisol on gluconeogenesis and glycogenolysis (Martinez-Porchas et al. 2009). An elevation of blood GLU may signify an escalating need for energy in response to chronic stress (Barton et al. 1986), such as activities associated with spawning. Plasma GLU concentrations in the current study were not significantly different between *Ichthyophonus*-negative and positive fish, with values around 2.5 mg/mL, while blood serum GLU concentrations were lower (~ 1.4 mg/mL) in spawning rainbow trout (Miller et al. 1983). Similarly, sockeye salmon on their

spawning grounds had lower concentrations of plasma cortisol and GLU than would be expected during terminal spawning and after prolonged fasting; this was attributed to adrenal exhaustion (Hruska et al. 2010). Muscle catabolism supports hepatic gluconeogenesis throughout the spawning migration, while metabolic changes and environmental triggers during spawning deplete these accumulated glycogen stores (French et al. 1983, Miller et al. 2009). The overall higher blood plasma GLU concentrations measured in the current study may reflect greater energy reserves obtained by Chinook salmon in the Yukon River drainage *prior* to river entry, higher glucogenic amino acid catabolism and thus higher glycogen stores, a relatively early time in spawning activities, or a combination of factors. Chronic stress exhibited during the long spawning migration may eventually reduce the concentration of cortisol in plasma, while also raising GLU concentrations as seen in this study, masking any potential difference as a result of infection with *Ichthyophonus*.

Chinook salmon that spawn in Yukon River tributaries travel over 1600 km to their spawning areas (Healey 1991). Blood enzyme levels fluctuate throughout the duration of their spawning migration (Wagner and Congleton 2004), with AST levels reaching their maximum levels during spawning (Hlavová 1989). However, as Chinook salmon reach their terminal spawning grounds, they are undergoing a down-regulation of protein biosynthesis and enzyme activity and continued down-regulation of muscle proteins and most glycolytic enzymes (Hane et al. 1966, Miller et al. 2009). Aspartate aminotransferase is a vitamin B-6 dependent enzyme that is important for amino acid metabolism (Ford et al. 1980) and is found in liver, heart, and skeletal muscle (Ford et al. 1980). Although female salmonids generally have higher levels of AST (Shahsavani et al. 2010), we did not detect a significant difference between males and females in the current study. We showed that *Ichthyophonus*-negative fish had significantly higher AST levels compared with *Ichthyophonus*-positive fish. Concentrations of AST in farmed Atlantic salmon were 202-351 U/L (Sandnes et al. 1988), similar to the *Ichthyophonus*-positive fish in this study, while AST in *Ichthyophonus*-negative fish was more than four times higher (Table 3.1). Similarly, Nile tilapia *Oreochromis niloticus* affected with nephrocalcinosis also had lower AST concentrations than healthy fish, which was attributed with disease progression (Chen et al. 2003). In contrast, Marty et al. (1998) found significantly higher AST levels in *Ichthyophonus*-positive Pacific herring *Clupea pallasii* due to severe inflammation. The same effect may not have been seen in the current study due to decreased enzyme activity at the end of a long spawning migration. It is also

possible that deficiency in zinc or magnesium, as well as vitamin B-12 as a direct result of inflammation (John and Mahajan 1979), can downregulate AST activity (Yousaf and Powell 2012), thus explaining lower levels of inflammation indicators in *Ichthyophonus*-positive fish, particularly at the end of spawning migration. This could be explored using quantitative ELISA kits targeting vitamins, such as B-12, and Inductively Coupled Plasma Mass Spectrometry to detect metals.

Creatine kinase (CK) is an enzyme that catalyzes the conversion of creatine to create phosphocreatine, while using energy in the form of adenosine triphosphate (ATP). This reaction is reversible and is therefore important for the regeneration of cellular ATP (Baird et al. 2012). CK has been used as a bioindicator of damage to CK rich tissues, such as the heart, skeletal muscle, brain, and spermatozoa, and is also an indicator of cardiac infarction (Baird et al. 2012). In the current study, CK had large standard deviations and was either present or not measurable. As a result, we found no difference in CK between disease presence or between sexes. It is, however, interesting, that all *Ichthyophonus*-positive fish had no detectable levels of CK, while *Ichthyophonus*-negative fish had a wide range of concentrations. These results could indicate that diseased fish are experiencing a range of enzyme depletion, which could be caused by many things, such as blood pH or temperature shift. Reduced CK activity has been observed in patients with liver disease, connective tissue disease, and rheumatoid arthritis, and reflects the overall decline in muscle mass and reduced physical activity (Stucki et al. 1996, Rosalki 1998). A decline in muscle mass after the long spawning migration could be a reasonable explanation for low CK levels in some individuals, especially *Ichthyophonus*-positive salmon. However, we found no significant difference in muscle protein of *Ichthyophonus*-positive and negative salmon (Chapter 2). Reduced physical activity (and associated low CK) in salmon after reaching the spawning ground is a possibility as well, and the non-detectable enzyme activities found in *Ichthyophonus*-positive fish could indicate them being moribund. Similar to our study, Chen et al. (2003) found that diseased tilapia had significantly lower CK enzyme activity compared with healthy controls. However, CK levels in healthy lake trout *Salvelinus namaycush* were on average 2315.8±1401.9 U/L (Edsall 1999), which is higher than the values detected in the current study (Table 3.1). These results also show that there can be high variability in CK activity. Fish exposed to low environmental oxygen concentrations had increased levels of CK to make ATP available to anoxic muscles (Myers et al. 1985, Van Waarde et al. 1990). It is therefore possible

that salmon in the current study with elevated levels of CK experienced anoxic conditions in their musculature due to exercise associated with the long migration. At the end of their spawning migration, fish may not be able to maintain high blood oxygen levels, thereby driving other enzymes, such as CK to higher levels. Creatine kinase (CK) levels can also be high during spawning (Hlavová 1989), and as mentioned previously, spawning activities depend on accumulated glycogen stores (French et al. 1983, Miller et al. 2009). The reliance on glycogen in turn indicates anaerobic metabolism (Pagnotta and Milligan 1991). During burst-type exercise in fish, muscles rely almost entirely on anaerobic metabolism of glycogen (Pagnotta and Milligan 1991). Overall, physiological changes associated with spawning and the spawning migration may confound any differences due to disease in these fish. Alternatively, severely diseased fish may be experiencing pre-spawning mortality and do not reach their final spawning grounds.

Another important enzyme found in blood plasma is ALP. It is responsible for removing phosphate groups from molecules and is concentrated in liver and kidney (Coleman 1992). This enzyme functions to hydrolyze phosphorylcholine, so that choline can be transported across the bile canalicular membrane. Values of ALP in the current study were not significantly different between *Ichthyophonus*-negative and *Ichthyophonus*-positive spawning Chinook salmon, but both groups had low levels compared with values of other salmonids (Folmar 1993). However, ALP levels of spawning female Chinook salmon were nearly twice as high as males. Similar to Atlantic salmon, high ALP levels in females could be an indicator of ovarian development and increased hepatic enzyme activity, although levels declined post-spawning for Atlantic salmon (Johnston et al. 1994).

Physiological changes associated with Chinook salmon spawning migration cause whole body changes that are important to consider. Maternal body condition at the time of spawning can affect the quality of salmon offspring (Bromage et al. 1992). Steroids, such as cortisol, are taken up by developing oocytes and may affect the developing eggs (Fevolden et al. 1991, de Jesus et al. 1993, Hwang and Wu 1993, Eriksen et al. 2006). Stressed fish produce eggs with higher cortisol concentrations, and this can hinder growth and development of the oocyte (Pankhurst and Van Der Kraak 1997). However, as described previously, females in general have higher cortisol concentrations (Idler et al. 1959, Folmar 1993), and there could be a built-in protective mechanism against the transfer of maternal steroids (Schreck et al. 2001), particularly in

salmonids where chronic stress is part of their life history. Mechanisms, such as regulation of substance transfer, from the mother to the egg and controlling timing of reproduction may have been developed to buffer eggs from deleterious effects of stressors, such as migration (Schreck et al. 2001). Alternatively, lipid deposition in the eggs may occur before cortisol concentrations of the female are elevated. Increased cortisol levels in offspring can reduce the appetite of juveniles and decelerate growth (Gregory and Wood 1999). In the current study, we did not detect any differences in whole body clinical chemistry parameters of alevins from *Ichthyophonus*-positive and negative parental Chinook salmon. This result is not completely unexpected, as we did not find any differences in blood plasma chemistry in spawning adult Chinook salmon between *Ichthyophonus*-positive and negative fish, with the exception of AST. Cortisol concentrations in healthy, unstressed juvenile Chinook salmon were near zero (Strange et al. 1978), which is similar to the results of this study (Table 3.3).

In the current study, there are several caveats to take into consideration. Most importantly, there was a limited sample size available for analysis. Samples obtained during this study relied on adequate escapement to the Salcha River spawning tributary and associated permission to sample. Yukon River Chinook salmon returns have been below expectations for several years, therefore in 2011 and 2012, we were not able to obtain independent samples separate from ADF&G. In addition, *Ichthyophonus* prevalence during the study years was low, and combined with an overall low availability of salmon led to sample size limitation in the *Ichthyophonus*-positive group. A larger sample size may have enabled us to better describe variability associated with spawning as well as disease related changes in blood chemistry parameters. Further, sampling *Ichthyophonus*-infected fish on the spawning grounds may inadvertently select for the successfully surviving or less affected part of the salmon population, while other fish infected with the parasite may die during their migration.

This study has shown that infection with *Ichthyophonus* does not appear to influence blood plasma chemistry parameters of Chinook salmon or the resulting alevins on the Salcha River. However, physiological changes and stress associated with spawning may confound any effects as a result of *Ichthyophonus* infection. It is likely that Chinook salmon females may have a protective mechanism that shields the eggs (and resulting offspring) from any deleterious changes in plasma chemistry connected with spawning. The migration stress imposed on Chinook salmon

during their freshwater spawning migration may be greater than the effects of disease, therefore masking any effects caused by *Ichthyophonus*.

3.6 Acknowledgements

We thank ADF&G for their cooperation with this project. This project was supported by the Pollock Conservation Cooperative Research Center through a grant awarded to L. Horstmann-Dehn. Fish resource permits were issued by ADF&G for the sampling years of 2010-2012 as follows: SF2010-129, SF2011-068, and SF2012-071, respectively. This study was authorized by the University of Alaska Fairbanks Institutional Animal Care and Use Committee under permit #170553-3.

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Table 3.1: Plasma chemistry of spawning Chinook salmon. Plasma chemistry parameters of spawning Chinook salmon (*Ichthyophonus*-positive and negative) sampled from the Salcha River, 2010 – 2012. A Kruskal-Wallis test was used and a p-value ≤ 0.05 was considered significant, (given in bold), H_0: Mean ranks of the two populations are equal. For plasma parameters that did not have any variation in value, NA replaces the range for that parameter. Literature values for plasma or serum of teleost fishes are provided for comparison.

	***Ichthyophonus*-positive** $n = 7$ **(3 females, 4 males)**	***Ichthyophonus*-negative** $n = 81$ **(39 females, 42 males)**	***p*-value**	**Literature Values for Teleosts**
Albumin [g/mL]				
Mean±1SD	0.04±0.01	0.03±0.01	0.17	0.018-0.024 Folmar (1993) Atlantic Salmon
Median	0.04	0.03		
Range	0.03-0.05	0.01-0.07		
Alkaline Phosphatase [U/L]				
Mean±1SD	100.7±14.5	95.0±83.5	0.14	100.0-300.0
Median	102.5	77.0		Folmar (1993)
Range	76.0-114.0	12.0-702.0		Rainbow Trout
Alanine aminotransferase [U/L]				
Mean±1SD	324.2±190.5	278.6±350.9	0.23	74.7-118.8
Median	302.5	186.0		Chen et al. (2003)
Range	109.0-587.0	2.5-2000.0		Nile Tilapia
Amylase [U/L]				
Mean±1SD	9.8±5.5	14.8±9.7	0.20	819.0
Median	9.0	13.0		Gu et al. (2013)
Range	2.5-18.0	0.0-55.0		Atlantic Salmon
Aspartate Aminotransferase [U/L]				
Mean±1SD	227.7±557.7	1043.3±865.5	**0.03**	202-351
Median	0.0	1364.0		Folmar (1993)
Range	0.0-1366.3	0.0-2192.0		Atlantic Salmon
Bile Acids [umol/L]				
Mean±1SD	17.5±0.0	17.5±0.0	1.00	21.0
Median	17.5	17.5		Gu et al. (2013)
Range	NA	NA		Atlantic Salmon
Blood Urea Nitrogen [mg/mL]				
Mean±1SD	0.03±0.03	0.03±0.02	0.93	0.11
Median	0.03	0.03		Davidson et al. (2014)
Range	0.01-0.08	0.00-0.16		Rainbow Trout

Table 3.1 continued

	Ichthyophonus-positive $n = 7$ (3 females, 4 males)	*Ichthyophonus*-negative $n = 81$ (39 females, 42 males)	*p*-value	Literature Values for Teleosts
Total Calcium [mg/mL]				
Mean±1SD	0.13±0.02	0.12±0.02	0.21	0.53
Median	0.13	0.12		Hasler et al. (2011)
Range	0.10-0.16	0.08-0.16		Chinook Salmon
Creatine Kinase [U/L]				
Mean±1SD	0.0±0.0	1041.1±2678.2	0.27	900-4100
Median	0.0	0.0		Folmar (1993)
Range	NA	0.0-9826.0		Striped Mullet
Cortisol [ng/mL]				
Mean±1SD	390.0±430.0	560.0±440.0	0.29	297.0
Median	310.0	440.0		McConnachie et al. (2012)
Range	3.0-104.0	50.0-174.0		Pink Salmon
Creatinine [mg/mL]				
Mean±1SD	0.001±0.0	0.001±0.0008	0.93	0.005
Median	0.001	0.001		Sandnes et al. (1988)
Range	NA	0.000-0.006		Atlantic Salmon
Globulin [g/mL]				
Mean±1SD	0.010±0.007	0.010±0.008	0.34	0.034
Median	0.020	0.010		Lepic et al. (2014)
Range	0.002-0.020	0.000-0.030		Vimba Bream
Glucose [mg/mL]				
Mean±1SD	2.62±0.95	2.59±1.14	0.18	0.3-0.8
Median	2.80	2.47		Folmar (1993)
Range	1.10-3.50	0.32-5.87		Rainbow Trout
Potassium [mmol/L]				
Mean±1SD	5.6±2.3	4.7±2.8	0.11	3.65
Median	4.8	4.6		Hasler et al. (2011)
Range	3.4-8.5	0.0-8.5		Chinook Salmon
Sodium [mmol/L]				
Mean±1SD	133.5±25.0	114.9±35.9	0.07	165.3
Median	130.0	122.5		Hasler et al. (2011)
Range	112.0-180.0	1.0-180.0		Chinook Salmon

Table 3.1 continued

	Ichthyophonus-positive $n = 7$ (3 females, 4 males)	*Ichthyophonus*-negative $n = 81$ (39 females, 42 males)	*p*-value	Literature Values for Teleosts
Phosphorous [mg/mL]				
Mean±1SD	0.15±0.05	0.12±0.04	0.79	0.61
Median	0.13	0.11		Hasler et al. (2011)
Range	0.11-0.25	0.06-0.26		Chinook Salmon
Uric Acid [mg/mL]				
Mean±1SD	0.01±0.003	0.02±0.002	0.71	0.006
Median	0.02	0.02		Haman et al. (2012)
Range	0.01-0.02	0.01-0.02		Atlantic Sharpnose
Total Bilirubin [mg/mL]				
Mean±1SD	0.006±0.002	0.007±0.009	0.82	0.003-0.080
Median	0.050	0.006		Folmar (1993)
Range	0.004-0.008	0.002-0.070		Rainbow Trout
Total Protein [g/mL]				
Mean±1SD	0.04±0.01	0.04±0.01	0.38	0.05
Median	0.04	0.05		Hasler et al. (2011)
Range	0.03-0.05	0.03-0.08		Chinook Salmon

Table 3.2: Plasma chemistry of *Ichthyophonus*-negative male and female spawning Chinook salmon. Plasma chemistry parameters of *Ichthyophonus*-negative male and female spawning Chinook salmon from the Salcha River, 2010 – 2012. A Kruskal-Wallis test was used and a *p*-value ≤ 0.05 considered significant (given in bold), H_0: Mean ranks of the two populations are equal. For plasma parameters that did not have any variation in value, NA replaces the range for that parameter.

	Male ***n* = 42**	**Female** ***n* = 39**	***p*-value**
Albumin [g/mL]			
Mean±1SD	0.003±0.001	0.004±0.001	**0.0004**
Median	0.003	0.004	
Range	0.001-0.005	0.002-0.007	
Alkaline Phosphatase [U/L]			
Mean±1SD	66.1±36.2	126.0±101.0	**0.0003**
Median	53.0	101.0	
Range	12.0-184.0	56.0-702.0	
Alanine Aminotransferase [U/L]			
Mean±1SD	293.5±396.9	269.4±277.0	0.74
Median	192.0	203.0	
Range	34.0-2000.0	2.5-1526.0	
Amylase [U/L]			
Mean±1SD	14.4±7.1	14.5±11.6	0.33
Median	15.0	12.0	
Range	2.5-29.0	0.0-55.0	
Aspartate Aminotransferase [U/L]			
Mean±1SD	1182.8±790.9	786.7±911.5	0.12
Median	1481.5	0.0	
Range	0.0-2131.0	0.0-2192.0	
Bile Acids [umol/L]			
Mean±1SD	17.5±0.0	17.5±0.0	1.00
Median	17.5	17.5	
Range	NA	NA	
Blood Urea Nitrogen [mg/mL]			
Mean±1SD	0.03±0.02	0.04±0.03	0.24
Median	0.03	0.03	
Range	0.01-0.07	0.0-0.2	
Total Calcium [mg/mL]			
Mean±1SD	0.12±0.02	0.12±0.02	0.96
Median	0.12	0.11	
Range	0.09-0.16	0.08-0.16	

Table 3.2 continued

	Male *n* = 42	Female *n* = 39	*p*-value
Cortisol [ng/mL]			
Mean±1SD	39.5±34.9	68.0±46.7	**0.01**
Median	31.8	55.2	
Range	4.9-141.0	0.3-174.2	
Creatine Kinase [U/L]			
Mean±1SD	1169.7±2941.4	761.3±2196.9	0.28
Median	0.0	0.0	
Range	0.0-9826.0	0.0-8725.0	
Creatinine [mg/mL]			
Mean±1SD	0.001±0.005	0.001±0.009	0.20
Median	0.001	0.001	
Range	0.0-0.003	0.0-0.006	
Globulin [g/mL]			
Mean±1SD	0.01±0.08	0.05±0.2	0.27
Median	0.01	0.09	
Range	0.0-0.03	0.0-1.5	
Glucose [mg/mL]			
Mean±1SD	2.6±1.1	2.6±1.2	0.78
Median	2.4	2.5	
Range	0.3-5.0	0.3-5.9	
Potassium [mmol/L]			
Mean±1SD	5.2±2.8	4.3±2.7	0.11
Median	5.8	4.3	
Range	0.0-8.5	0.0-8.5	
Sodium [mmol/L]			
Mean±1SD	121.7±26.0	110.3±42.7	0.44
Median	124.0	121.0	
Range	50.0-180.0	1.0-180.0	
Phosphorous [mg/mL]			
Mean±1SD	0.13±0.046	0.12±0.033	0.65
Median	0.12	0.12	
Range	0.055-0.26	0.063-0.19	
Uric Acid [mg/mL]			
Mean±1SD	0.01±0.05	0.01±0.03	0.93
Median	0.01	0.01	
Range	0.01-0.02	0.01-0.02	

Table 3.2 continued

	Male ***n*** **= 42**	**Female** ***n*** **= 39**	***p*-value**
Total Bilirubin [mg/mL]			
Mean±1SD	0.006±0.003	0.009±0.011	0.13
Median	0.005	0.007	
Range	0.002-0.001	0.002-0.007	
Total Protein [g/mL]			
Mean±1SD	0.05±0.01	0.04±0.01	0.33
Median	0.05	0.04	
Range	0.03-0.06	0.03-0.06	

Table 3.3: Total body homogenate clinical chemistry parameters for alevin. Total body homogenate clinical chemistry parameters for alevins sired by *Ichthyophonus*-positive and *Ichthyophonus*-negative Chinook salmon sampled 2010 – 2012. A Kruskal-Wallis test was used and a *p*-value ≤ 0.05 considered significant, H_0: Ranks of the two populations are equal. For clinical chemistry parameters that did not have any variation in value, NA replaces the range for that parameter.

	***Ichthyophonus*-positive** ***n* = 5**	***Ichthyophonus*-negative** ***n* = 30**	***p*-value**
Albumin [g/mL]			
Mean±1SD	0.005±0.001	0.005±0.001	0.93
Median	0.005	0.005	
Range	0.002-0.005	0.002-0.005	
Alkaline Phosphatase [U/L]			
Mean±1SD	2.5±0.0	2.2±0.8	0.14
Median	2.5	2.5	
Range	NA	0.0-2.5	
Alanine Aminotransferase [U/L]			
Mean±1SD	30.9±14.7	30.5±12.7	0.65
Median	28.0	26.3	
Range	17.3-58.0	14.7-57.3	
Amylase [U/L]			
Mean±1SD	2.1±0.9	2.3±0.7	0.68
Median	2.5	2.5	
Range	0.0-2.5	0.0-2.5	
Aspartate Aminotransferase [U/L]			
Mean±1SD	62.5±38.8	51.4±21.6	0.37
Median	42.7	44.0	
Range	38.5-144.0	32.1-117.7	
Bile Acids [umol/L]			
Mean±1SD	17.5±0.0	17.5±0.0	1.00
Median	17.5	17.5	
Range	NA	NA	
Blood Urea Nitrogen [mg/mL]			
Mean±1SD	0.01±0.0	0.01±0.0	1.00
Median	0.01	0.01	
Range	NA	NA	
Total Calcium [mg/mL]			
Mean±1SD	0.02±0.00	0.02±0.00	1.00
Median	0.02	0.02	
Range	NA	NA	

Table 3.3 continued

	Ichthyophonus-positive *n* = 5	*Ichthyophonus*-negative *n* = 30	*p*-value
Cortisol [ng/mL]			
Mean±1SD	0.9±0.4	0.9±0.7	0.66
Median	0.9	0.8	
Range	0.3-1.0	0.3-4.0	
Creatine Kinase [U/L]			
Mean±1SD	3342.0±1556.8	5900.1±4162.4	0.48
Median	3146.8	4273.3	
Range	2119.8-11097.3	2681.3-14462.0	
Creatinine [mg/mL]			
Mean±1SD	0.001±0.000	0.001±0.000	1.00
Median	0.001	0.001	
Range	NA	NA	
Globulin [g/mL]			
Mean±1SD	0.0±0.0	0.0±0.0	1.00
Median	0.0	0.0	
Range	NA	NA	
Glucose [mg/mL]			
Mean±1SD	0.40±0.09	0.46±0.16	0.68
Median	0.39	0.44	
Range	0.27-0.59	0.28-0.80	
Potassium [mmol/L]			
Mean±1SD	6.9±2.0	5.6±1.8	0.13
Median	6.9	4.9	
Range	4.7-10.5	3.0-8.9	
Sodium [mmol/L]			
Mean±1SD	180.0±0.0	180.0±0.0	1.00
Median	180.0	180.0	
Range	NA	NA	
Phosphorous [mg/mL]			
Mean±1SD	0.48±0.16	0.58±16.40	0.17
Median	0.45	0.50	
Range	0.30-0.83	0.29-0.89	
Uric Acid [mg/mL]			
Mean±1SD	0.015±0.006	0.014±0.002	0.82
Median	0.014	0.014	
Range	0.011-0.018	0.012-0.018	

Table 3.3 continued

	Ichthyophonus-positive *n* = 5	*Ichthyophonus*-negative *n* = 30	*p*-value
Total Bilirubin [mg/mL]			
Mean±1SD	0.002±0.004	0.002±0.002	0.52
Median	0.002	0.002	
Range	0.002-0.003	0.002-0.003	
Total Protein [g/mL]			
Mean±1SD	0.010±0.001	0.010±0.001	0.94
Median	0.010	0.010	
Range	0.007-0.01	0.005-0.01	

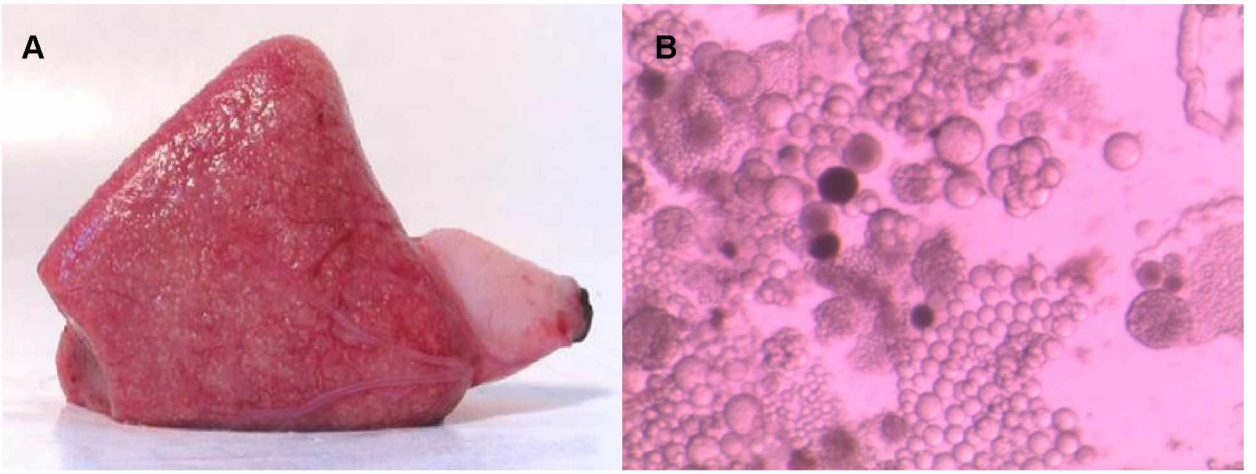

Figure 3.1: Clinical signs of *Ichthyophonus* in Chinook salmon. Clinical signs of *Ichthyophonus* infection in the heart of a spawning Chinook salmon (A); white diffuse lesions are visible. (B) *Ichthyophonus* spores at 50x magnification. Photos by L. Horstmann-Dehn (A) and C. Whipps (B).

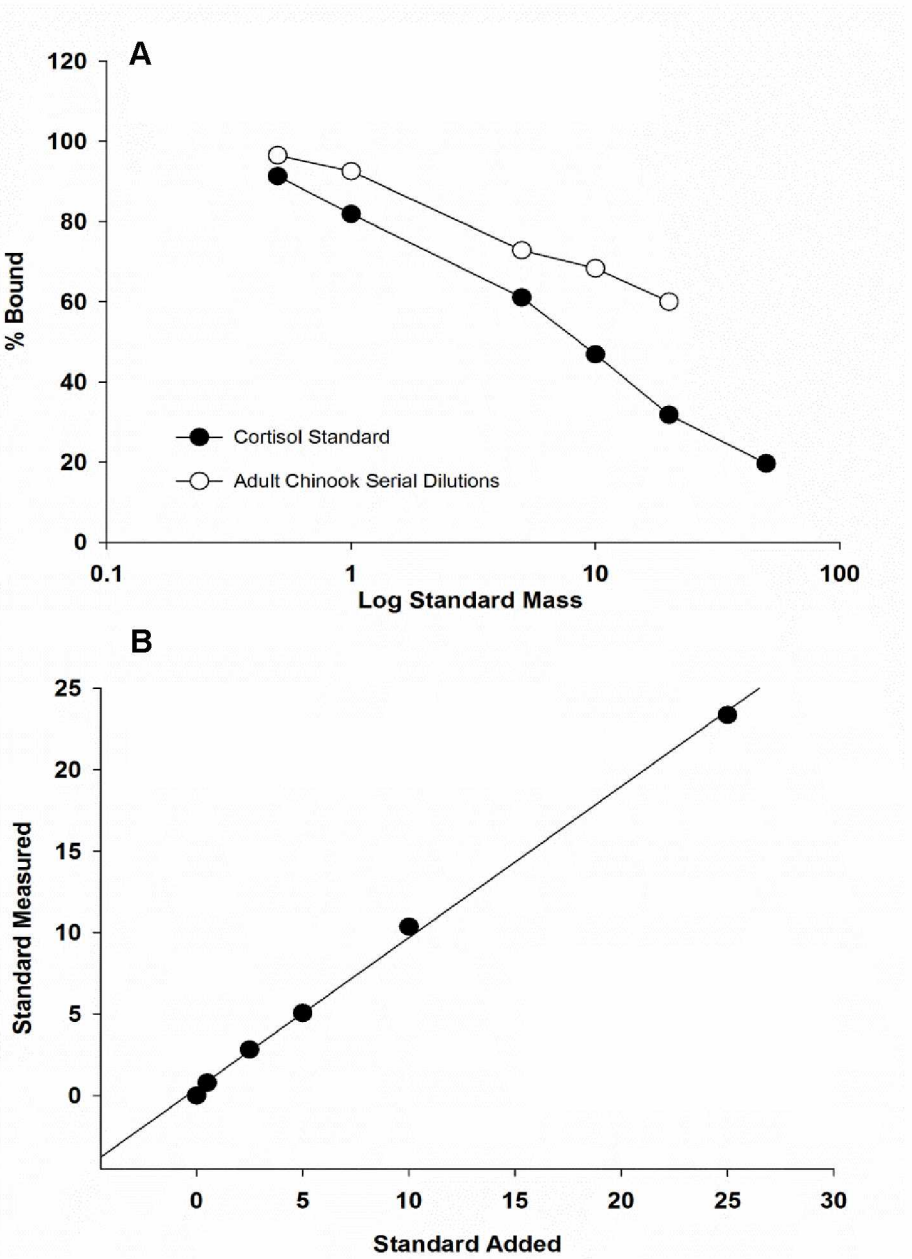

Figure 3.2: Validation of radioimmunoassay for spawning Chinook salmon plasma cortisol. (A) Serial dilutions of cortisol (% binding) from neat to 1:16 pooled spawning Chinook salmon plasma (open circle), plotted with a cortisol kit standard (closed circle) versus log-standard mass [ng/mL] indicating displacement by the pool parallel to that of the standard. (B) Chinook salmon plasma cortisol [ng/mL] added 1:1 to each of the assay standards [ng/mL], with a slope of 0.93 (R^2=0.99) indicating a 93 % accuracy of the assay in the measurement of Chinook salmon plasma cortisol.

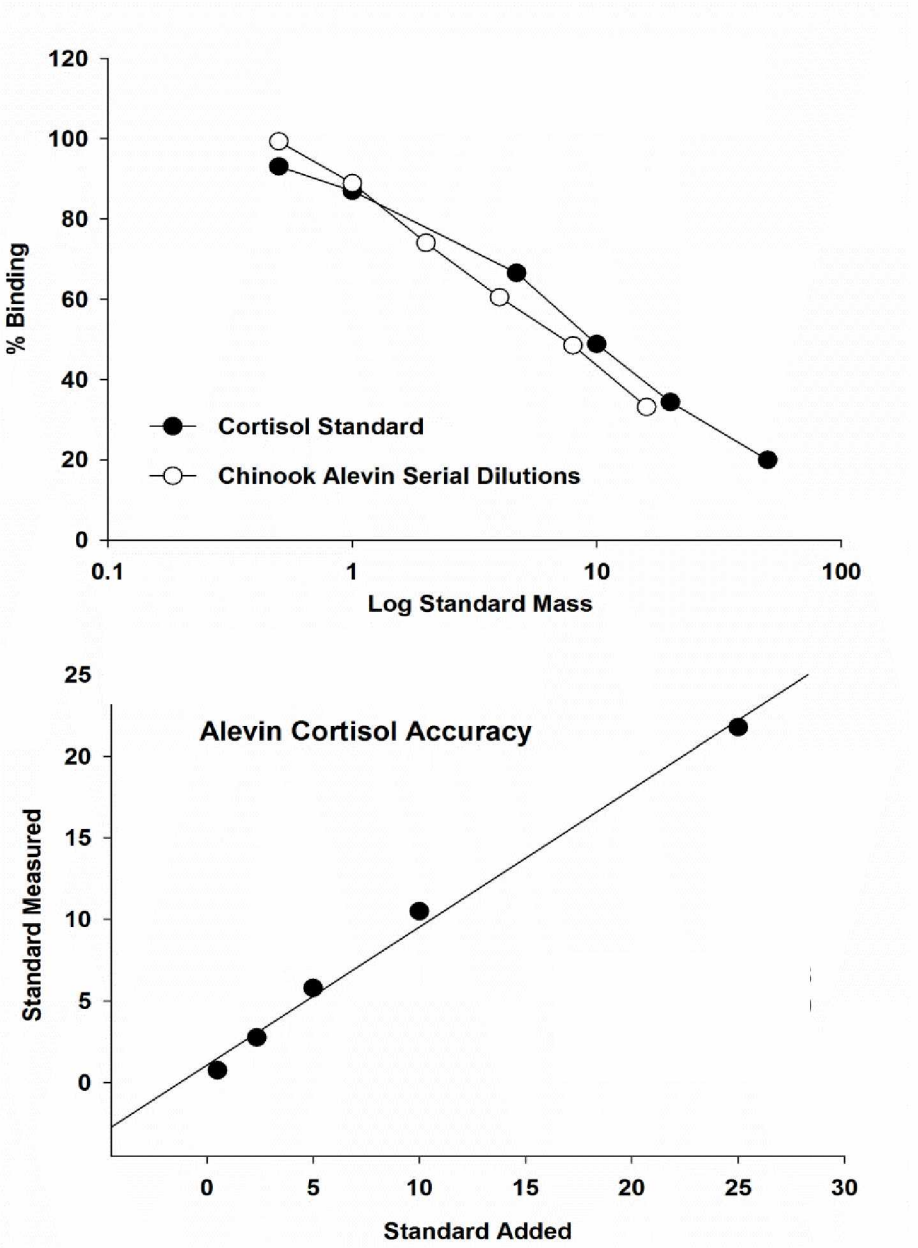

Figure 3.3: Validation of radioimmunoassay for Chinook salmon alevin body homogenates. (A) Serial dilutions of cortisol (% binding) from neat to 1:16 pooled Chinook salmon alevin total body homogenates (open circle) plotted with a cortisol kit standard (closed circle) versus log-standard mass [ng/mL] indicating displacement by the pool parallel to that of the standard. (B) Chinook salmon alevin cortisol of total body homogenates [ng/mL] added 1:1 to each of the assay standards [ng/mL], with a slope of 0.85 (R^2=0.99) indicating a 85 % accuracy of the assay in the measurement of Chinook salmon alevin cortisol of total body homogenates.

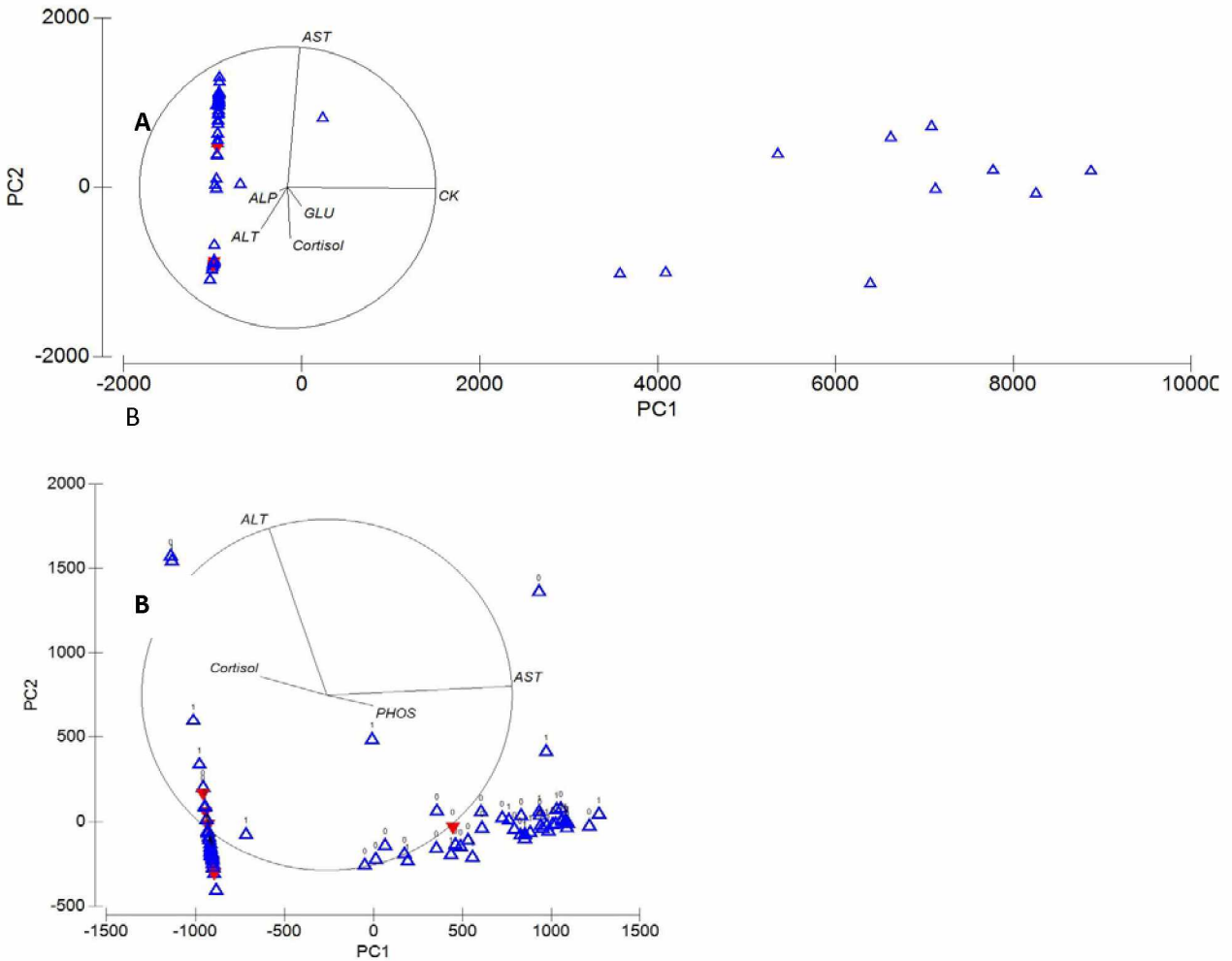

Figure 3.4: Principal components analysis of blood plasma parameters of spawning Chinook salmon. A) Principal components analysis (PCA) of blood plasma parameters of spawning Chinook salmon: albumin, alkaline phosphatase, alanine aminotransferase (ALT), amylase, aspartate aminotransferase (AST), bile acids, blood urea nitrogen, calcium, creatine kinase (CK), cortisol, creatinine, globulin, glucose, potassium, sodium, phosphorous (PHOS), uric acid, total bilirubin, and total protein. Red triangles represent *Ichthyophonus*-positive Chinook salmon and blue triangles show *Ichthyophonus*-negative fish. PC1 explains 88.0 % of the variability, and PC2 accounts for 10.2 % for a total of 98.2 % the explained variability. Vectors for select variables are shown (black lines), and vector length indicates the importance of the variable contribution. (B) PCA of blood plasma parameters of spawning Chinook salmon, excluding CK. Red triangles represent *Ichthyophonus*-positive Chinook salmon, and blue triangles show *Ichthyophonus*-negative fish, with "1" indicating female and "0" male fish. PC1 explains 84.5 % of variability and PC2 explains 13.0 % of variability, totaling 97.4 % of the explained variability.

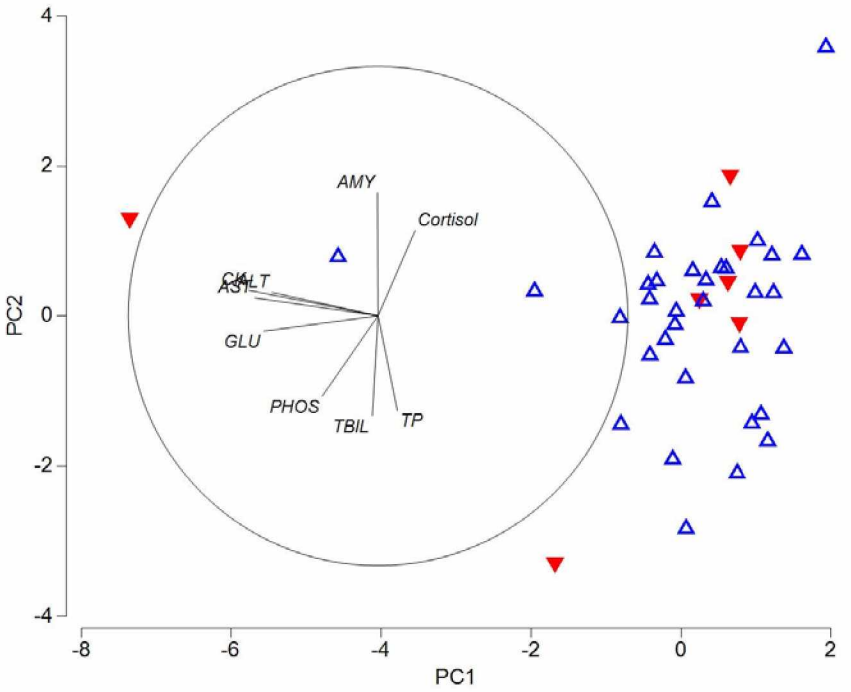

Figure 3.5: Principal components analysis of clinical diagnostic parameters of Chinook salmon alevins. Principal components analysis (PCA) of clinical diagnostic parameters of Chinook salmon alevin total body homogenates: albumin, alkaline phosphatase, alanine aminotransferase (ALT), amylase (AMY), aspartate aminotransferase (AST), bile acids, blood urea nitrogen, calcium, creatine kinase (CK), cortisol, creatinine, globulin, glucose (GLU), potassium, sodium, phosphorous (PHOS), uric acid, total bilirubin(TBIL), and total protein (TP). Red triangles represent alevins from *Ichthyophonus*-positive adults, and blue triangles show alevins from *Ichthyophonus*-negative parents. PC1 explained 22.4 % of variability, PC2 explained 36.1 % of variability for a total of 58.5 % variability explained. Vectors for select variables are shown (black lines), and vector length indicates the importance of the variable contribution.

Chapter 4: General Conclusion

Chinook salmon, *Oncorhynchus tshawytscha*, are an important resource in Alaska and are valued in subsistence, commercial, personal use, and sport fisheries. The people of Alaska rely on this annual influx of sustenance, and often, these fish are the only cash income for subsistence communities. Chinook salmon also provide food for marine mammals, predatory fishes, seabirds, and terrestrial animals (including humans), and contribute marine-derived nutrients to river banks after spawning (Cederholm et al. 1999). Chinook salmon returns to the Yukon River and its tributaries have declined substantially since the late 1990s (Schindler et al. 2013). The reason for these declines is not known. Potential causes include overfishing, bycatch, climate change, and disease. *Ichthyophonus* is a marine-derived parasite and was identified in the 1980s in Chinook salmon in the Yukon River (Kocan et al. 2004). The prevalence of *Ichthyophonus* in Yukon River Chinook salmon appears to be inversely correlated with their population abundance (Zuray et al. 2012). Evidence suggests that *Ichthyophonus* may cause pre-spawning mortality in Chinook salmon (Kocan et al. 2004, 2009, Kocan and Hershberger 2006), but other physiological effects have also been described in other species, such as higher lipid and protein loss compared with healthy fish (Vollenweider et al. 2011). Overall, fish infected with *Ichthyophonus* are not thought to recover and will eventually succumb to the disease (Sindermann 1965; Marty et al. 1998, Kocan et al. 1999, Hershberger et al. 2002). In addition, physiological response to parasites, as well as parasite load, are temperature dependent in poikilotherms (Finn and Nielson 1971, Okamoto et al. 1987, Kocan et al. 2009). Northern ecosystems are undergoing rapid change and marine water temperatures have been projected to increase by approximately 2 °C (Wang et al. 2010), while in-river water temperatures on the Yukon River in June have increased by 2.5 °C over the past 30 years (Kocan et al. 2004, 2009, Kahler et al. 2007). The combination of changing environmental conditions and the potential for *Ichthyophonus* to not only cause substantial pre-spawning mortality (Kocan et al. 2004, 2006), but also induce emaciation and thus reduced fecundity/offspring quality (e.g., Kurita et al. 2003), make disease effect studies in Yukon River Chinook salmon important.

This study used a combination of proximate composition and blood chemistry analyses to compare *Ichthyophonus*-infected and non-infected spawning Chinook salmon and their resulting eggs and alevins. Proximate composition analysis (via lipid, protein, and energy content)

provided a means to determine body condition of spawning Chinook salmon, as well as quality of eggs and alevins. Blood chemistry and hormone analyses were used to investigate the physiological state of Chinook salmon, by examining inflammation and stress response indicators in both spawning adults and alevins. The combination of these techniques allowed us to examine the direct effects of *Ichthyophonus* on spawning adults, and potentially propagated effects on the second generation. *Ichthyophonus* is associated with reduced body condition and emaciation (Kramer-Schadt et al. 2010, Vollenweider et al. 2011), and maternal condition and egg lipid affect growth and survival of the offspring (Beacham and Murray 1990, Kamler 2005). It is therefore important to evaluate if *Ichthyophonus* can influence Chinook salmon populations on an indirect level. If disease has a measureable impact on the second generation, this information would have to be taken into account in spawner-recruit models.

In chapter 2, proximate composition parameters (% lipid, % crude protein, energy content) of *Ichthyophonus*-infected and non-infected spawning Chinook salmon and the resulting eggs and alevins were compared. We hypothesized that *Ichthyophonus* would have a measureable deleterious effect on the fish host, based on previous studies showing emaciation (Rahimian 1998, Kramer-Schadt et al. 2010). Contrary to our prediction, proximate composition of spawning adult salmon muscle tissue did not differ between *Ichthyophonus*-infected and non-infected fish. We may not have seen a condition difference caused by the parasite, due to fish being sampled at the end of their spawning migration, when most available body resources may have been utilized. It is therefore possible that changes in the body that occur as a result of spawning may overshadow any effects of disease. In the future, it will be important to assess body condition of *Ichthyophonus*-infected and non-infected salmon along their spawning migration. Fish with reduced body reserves as a result of infection with the parasite, may potentially die along the way. In addition, it will be important to address if salmon with different spawning destinations and length of travel (i.e., Canada-bound Chinook salmon versus Andreafsky River spawners) have differential survival probabilities as a result of declining body reserves, as has been suggested by Kocan et al. (2006). We further examined Chinook salmon egg proximate composition. We hypothesized that eggs from *Ichthyophonus*-infected Chinook salmon would have lower reserves compared with eggs from non-infected parents. Egg composition showed some differences, with lipid and water content of eggs from *Ichthyophonus*-infected Chinook salmon being unexpectedly higher relative to eggs from non-infected females. It is possible that eggs from *Ichthyophonus*-

infected fish, while higher in lipid content, may have different, potentially lower quality fatty acids and phospholipids that affect survival to hatching and resulting alevin success (Cowey et al. 1985). Once eggs hatched into alevins, all analyzed proximate composition parameters including morphometrics (i.e., weight, length, and yolk sac height) were similar between offspring from *Ichthyophonus*-infected and non-infected parents, indicating that offspring from infected parents were not at any obvious disadvantage. Another important consideration for egg quality is vitamin content; vitamin A (in the form of retinol and carotenes) is deposited by the female from muscle and liver and is then combined to egg yolk proteins that are essential for egg viability and normal development of embryos (Palace and Werner 2006). Future studies should examine vitamin content to explore if eggs and alevins from *Ichthyophonus*-infected parents show differences compared with non-infected parents. Further, future studies should examine behavior of offspring from *Ichthyophonus*-infected parents compared with offspring from healthy parents. It is possible that offspring from diseased parents experience other disadvantages, such as lower swimming stamina, slower reaction times, and thus higher predation rates (Mesa 1994, Mesa et al. 1998).

In this study, eggs and milt from *Ichthyophonus*-infected and non-infected parents were collected and cross-fertilized to create offspring of "healthy" parents, and those of infected females with "healthy" males, as well as infected males with "healthy" females. While the overall survival of fertilized eggs to hatching was not significantly different between offspring from *Ichthyophonus*-negative and positive parents, eggs of non-infected females fertilized with *Ichthyophonus*-positive milt yielded zero survival. The sample size in this study was limited, so any conclusions should be made with caution. However, this finding should be investigated in the future with more robust sample sizes. We then examined milt from *Ichthyophonus*-infected and non-infected males and found no difference in overall milt cell counts or the proportion of live sperm cells. However, other important parameters of milt quality should be taken into account in the future, such as antioxidants and lipid classification, specifically phospholipids and polyunsaturated fatty acids, which are crucial to the fertilizing ability of milt (Labbe et al. 1995, Bell et al. 1996, Pustowka et al. 2000, Asturiano et al. 2001, Dabrowski and Ciereszko 2001). Other important parameters to investigate in prospective studies should include sperm cell motility and energetics, antioxidant effects, heat-shock protein expression, gamete DNA damage, and associated potential fertilization disadvantages as a result of disease (e.g., Dabrowski and Ciereszko 2001, Rurangwa et al. 2004, Migaud et al. 2013). Overall, we did not find any differences in spawning Chinook

salmon or their resulting alevins. However, samples were only collected from fish that successfully made it to their final spawning grounds, therefore, we may have inadvertently selected only the hardiest survivors, leaving out individuals that may have experienced severe disease effects and pre-spawning mortality.

In chapter 3, blood chemistry parameters (e.g., alanine aminotransferase, cortisol, creatine kinase, glucose) of *Ichthyophonus* infected and non-infected spawning Chinook salmon and the resulting alevins were compared. Evaluating blood chemistry parameters of spawning Chinook salmon allowed us to determine if *Ichthyophonus*-infected fish had an elevated stress response or were at a physiological disadvantage compared with non-infected fish (Barton et al. 1986, Schreck et al. 1997, Marty et al. 1998, Yousaf and Powell 2012). We hypothesized that spawning Chinook salmon infected with *Ichthyophonus* would exhibit a stress response and subsequently have higher concentrations of cortisol and glucose compared with non-infected fish, as well as detectable increases in tissue inflammation and tissue damage indicators (e.g., alanine aminotransferase, aspartate aminotransferase, and creatine kinase). We did not find differences in most plasma chemistry parameters between spawning Chinook salmon with the exception of the tissue damage indicator aspartate aminotransferase, which was higher in non-infected salmon. While this outcome was unexpected, it is possible that either migration-related physiological changes or vitamin B-12 deficiency in diseased fish contributed to this result. Elemental deficiency (e.g., zinc, magnesium) as well as vitamin B-12 deficiency, as a direct result of disease-related inflammation, can lead to a down-regulation of aspartate aminotransferase (John and Mahajan 1979, Yousaf and Powell 2012). This possible correlation should be explored in more depth in future studies. Another surprising finding was the lack of stress response in *Ichthyophonus*-infected versus non-infected salmon. Adrenal exhaustion is a possible explanation, where the chronic production of cortisol (as part of the spawning migration) exhausts the adrenal gland (McConnachie et al. 2012). It will be important in upcoming studies to track stress response in Chinook salmon at river entry and along their migration to determine if cortisol concentrations indeed decline with increasing travel distance or at onset of spawning. Overall, disease may only have a minimal effect on blood plasma parameters, and changes associated with migration (e.g., cessation of feeding, muscle catabolism) are the main factors influencing plasma chemistry in spawning adults.

In this study, we hypothesized that some blood plasma parameters, such as lipophilic cortisol, would be transferred to the next generation, such that offspring produced by *Ichthyophonus*-positive parents would differ from those of *Ichthyophonus*-negative parents. However, all clinical diagnostic parameters were not different in alevins resulting from either infected or non-infected parents. This finding is not too surprising as adults did not differ by disease status. Considering the profound physiological and biochemical changes that adult salmon undergo as part of their migration, including muscle and hepatic catabolism (French et al. 1983, Benskin et al. 2014), it is possible that salmon have developed a system, where the eggs (and resulting alevins) are shielded from metabolic and endocrine spawning-related products of the mother (Schreck et al. 2001). In general, the results of this study indicate that juveniles do not suffer any deleterious effects from the maternally transferred metabolic compounds analyzed herein. However, fish produce hundreds of metabolites as a product of normal metabolic or physiological processes, and selection of just a few parameters may bias the outcome. Newer tools, such as metabolomic profiling, could be useful here, because they examine all occurring compounds simultaneously, have application in biomarker and disease pathway identification (Viant 2007), and could thus more easily distinguish between spawning or disease-associated changes (Benskin et al. 2014, Cipriano et al. 2015). Using the metabolomics approach, Nichols et al. (2012) were able to identify several compounds that are either up or down-regulated in marine Chinook salmon infected with *Ichthyophonus* compared with non-infected fish. Most of these compounds were involved in cell signaling and an immune response cascade that included phospholipids, ceramids, and fatty acids (Nichols et al. 2012). As mentioned above, lipids have important functions in growth and reproduction, and more detailed studies on disease-related changes in lipid classes and lipid allocation are needed.

In future studies, it would be important to incorporate scaling of disease severity caused by *Ichthyophonus*. Gross observation of epidermal ulcers and microscopic examination of tissues in the field are good techniques to determine disease severity as well as identify presence of the parasite (Kocan et al. 2011, Kent et al. 2013). For example, Whipps et al. (2005) established a ranking system of light to heavy infection in cardiac tissue by histologically observing the number of *Ichthyophonus* spores found in a sample of known size. An even more measurable technique to determine severity is quantitative polymerase chain reaction (qPCR), which can numerically quantify parasite DNA specific to *Ichthyophonus*. Validation for the use of qPCR in the

identification of *Ichthyophonus* has recently been completed (White et al. 2013), and would make inclusion of this method in future studies valuable. Measures of disease severity could then be correlated to the degree of physiological responses by the fish host, thus adding another level of understanding in the disease process and its consequences.

Forthcoming studies should consider the assessment of *Ichthyophonus* at all life stages of Chinook salmon. Juvenile Chinook salmon migrating out of the Yukon River are not infected with *Ichthyophonus,* but when adults return to spawn, a portion of the population is often infected (Kocan et al. 2004). This indicates that adults become infected with *Ichthyophonus* during the marine phase of their lifecycle, although *Ichthyophonus* prevalence in Chinook salmon during their marine phase was low (Nichols et al. 2012). While juveniles are not infected with *Ichthyophonus* during their freshwater outmigration, or infection severity is too low to detect the parasite with histological methods (Kocan et al. 2004), it is nonetheless possible that parental infection affects juvenile survival in the marine environment. Chinook salmon spend the majority of their life in their marine stage. Therefore, it is important to determine the effects of the parasite during this extended life stage, and pinpoint where the main source of infection stems from. *Ichthyophonus* has been positively identified in the copepod *Calanus finmarchicus* (Wolke 1975) and many other marine species. Torgersen et al. (2002) also reported an *Ichthyophonus*-like pathogen in *Calanus* spp. that changes the coloration of the infected copepod, and consequently make it an easy prey target. It is therefore important to understand the marine reservoir species of *Ichthyophonus*, and further consider the effect of climate variability in the Bering Sea to the distribution and abundance of this reservoir.

Management of Chinook salmon on the Yukon River often only incorporates the number, size, and ages of returning Chinook salmon as they migrate upstream to their spawning grounds, without consideration of quality of these fish or their gametes (Lewis et al. 2015). Analyses of spawner-recruit relationships are performed for the Yukon River and its tributaries using escapement and harvest data that are collected annually (Evenson 2002). Returning adult Chinook salmon abundance has been estimated yearly in the Salcha River since 1987 (Evenson 2002); however, while biological escapement goals, based on quantification of fish passage, have mostly been met, the overall number of returning fish to the Yukon River have continued to decline (ADF&G 2013, 2015, JTC 2013, 2014). This indicates that there are other factors that

need to be taken into account when considering the robustness of the population of Chinook salmon returning to the Yukon River. The Alaska Department of Fish and Game (ADF&G) uses an assortment of methods to determine numbers of fish returning to the Yukon River, including sonar, mark-recapture, and counting towers (Evenson 2002, Brannian et al. 2005). After fish have spawned and expired, carcass surveys are conducted on the spawning grounds to determine age composition of the returning Chinook salmon population, to generate estimates of total recreational, commercial, and subsistence fishery harvest, and to complete return estimates (Evenson 2002). This information is vital for maintaining important fisheries and achieving desired escapement levels (Evenson 2002); however, these methods do not account for the condition in which these fish reach their spawning grounds, or if they were able to successfully reproduce. Yet, the quality of Chinook salmon returning to the Yukon River is important to consider, because if fish making it to their final spawning grounds are not successfully spawning or are spawning inferior gametes, it will affect future returns to the river. In the current study, we did not find significant differences between *Ichthyophonus*-infected and non-infected spawning Chinook salmon and their offspring, therefore, our results do not necessarily warrant the incorporation of these qualitative methods. However, as mentioned above, several other factors can and should be considered for fish on the spawning grounds that could affect spawner quality and that of their offspring, in particular in the light of continued Chinook salmon run failures. It is important that scientists continue to explore bioindicators of disease, declining fecundity, and juvenile survival. In turn, it is essential that fisheries management starts to incorporate new findings into spawner-recruit models to assure healthy and robust Chinook salmon returns in the future.

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Appendix A: UAF IACUC approval letter, 2010

(907) 474-7800
(907) 474-5638 fax
fyiacuc@uaf.edu
www.uaf.edu/iacuc

Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

June 14, 2010

To: Trent Sutton, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [170553-3] Effect of Ichthyophonus infection on Yukon River Chinook salmon hatching success, growth, and survival

The IACUC reviewed and approved the New Project referenced below by Designated Member Review.

Received:	June 8, 2010
Approval Date:	June 14, 2010
Initial Approval Date:	June 14, 2010
Expiration Date:	June 14, 2011

This action is included on the July 1, 2010 IACUC Agenda.

The PI is responsible for acquiring and maintaining all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol, and could result in revocation of IACUC approval.

Appendix B: Alaska Department of Fish and Game permit, 2010

STATE OF ALASKA
DEPARTMENT OF FISH AND GAME
P.O. BOX 115525
JUNEAU, ALASKA 99811-5525

Permit #: **SF2010-129**
Expires: **8/31/2010**
Collections Report Due: **9/31/2010**

FISH RESOURCE PERMIT
(For Scientific/Educational Purposes)

This permit authorizes Larissa Dehn *(whose signature is required on page 2 for permit validation)*
person

of SFOS University of Alaska Fairbanks *agency or organization* **at** Box 757220, Fairbanks, AK 99775 *address*

to conduct the following activities **from** July 1, 2010 **to** August 31, 2010 in accordance with AS 16.05.930:

Purpose: To investigate the physiological cost of a parasitic infection in the target species.

Location: Chena/Salcha Rivers

Species Collected: King salmon

Method of Capture: Boat operated electrofishing in collaboration with ADF&G brood stock take

Final Disposition: **CAUTION: *This authorization depends upon run strength with ADF&G making the call.***
≤25 adult males and ≤25 adult females from each river may be collected and held with other ADF&G broodstock until ripe gametes and other biological samples are harvested and transported to a secure UAF lab for further research **(Stipulations #6 and #7)**
After gamete extraction, all salmon flesh of good quality should be donated to a local charity with the remaining carcasses treated as biological waste.

-Continued on Back-

COLLECTIONS REPORT DUE September 30, 2010. The report, using a data submission form furnished by ADF&G), shall include **ALL** species, numbers, dates, and locations of collection (datum/GPS coordinates in the decimal degrees format (dd.ddddd)) and disposition, and if applicable, sex, age, and breeding condition, and lengths and weights of fish handled. *It must also include the date/time the local biologist was contacted for final authorization to carry out collecting activities.* A completion report (abstract, background, methods, data, analysis), if not submitted with the collection report described above, must be submitted to the department by: ***February/2011***. Data from such reports are considered public information. The report shall also include other information as may be required under the permit stipulations section.

GENERAL CONDITIONS, EXCEPTIONS AND RESTRICTIONS

1. This permit must be carried by person(s) specified during approved activities who shall show it on request to persons authorized to enforce Alaska's fish and game laws. This permit is nontransferable and will be revoked or renewal denied by the Commissioner of Fish and Game if the permittee violates any of its conditions, exceptions or restrictions. No redelegation of authority may be allowed under this permit unless specifically noted.
2. No specimens taken under authority hereof may be sold or bartered. All specimens must be deposited in a public museum or a public scientific or educational institution unless otherwise stated herein. Subpermittees shall not retain possession of live animals or other specimens.
3. The permittee shall keep records of all activities conducted under authority of this permit, available for inspection at all reasonable hours upon request of any authorized state enforcement officer.
4. Permits will not be renewed until the department has received detailed reports, as specified above.
5. UNLESS SPECIFICALLY STATED HEREIN, THIS PERMIT DOES NOT AUTHORIZE the exportation of specimens or the taking of specimens in areas otherwise closed to hunting and fishing; without appropriate licenses required by state regulations; during closed seasons; or in any manner, by any means, at any time not permitted by those regulations.

Bob Pearson — Fish Resource Permit Coordinator, Division of Sport Fish

Robert Begtrup for Director, Division of Sport Fish — 5-28-10 Date

SF2010-129 ***continued (page 2 of 2)***

Authorized Personnel: The following persons may perform collecting activities under terms of this permit:

Shannon Atkinson, Sean Brennan, Larissa Dehn, Nicole, Farnham, Theresa Floyd, Trent Sutton, Brian Walker

Employees and volunteers under the direct supervision of, and in the presence of, one of the authorized personnel listed above may participate in collecting activities under terms of this permit.

Permit Stipulations:

1) The local Area Management Biologist (AMB), Audra Brase (459-7244; audra.brase@alaska.gov) Lower Tanana River, must be contacted for final authorization **prior** to you engaging in any collecting activities. *The time/date of this contact must be included in your collections report (using the "data submission form" furnished by ADF&G).* This AMB has the right to specify methods for collecting, as well as limiting the collections of any species by number, time and location.
2) Each piece of unattended sampling gear must be; 1) labeled with the permittee's name, telephone number, and permit number, 2) located with GPS coordinates, and 3) accounted for/removed at the conclusion of sampling.
3) If anadromous fish species new to permitted streams and rivers are found, the permit holder will work closely with ADF&G to see that information is included in the database for the *Catalog of Waters Important for Spawning, Rearing or Migration of Anadromous Fishes.* Anadromous fish include *Oncorhynchus spp.*, Arctic char, Dolly Varden, sheefish, smelts, lamprey, whitefish, and sturgeon. Please direct questions to J. Johnson, 267-2337 or j.johnson@alaska.gov
4) If samples suitable for genetic analysis are collected under this permit and are still in the permittee's possession, the permittee agrees to provide them to ADF&G within three months of a request.
5) **Atlantic salmon** and other **non-native invasive aquatic species** that you encounter during your sampling should be killed. In such an event please contact the nearest ADF&G office (**Stipulation # 1**) ASAP with species identification or description, capture location or location of sighting if capture is not possible, number captured, size, and sex. Preserve and turn in the whole specimen to the nearest ADF&G office.
6) This permit will fulfill the requirements of 5AAC 41.005 – 41.060 pertaining to fish transport permits (FTP's), with the condition that the transported species be destroyed when the permit expires and not be released to the wild.
7) All aquaria effluent must be treated before discharge. Fourteen ounces of household chlorine bleach must be added to and adequately mixed with each gallon of wastewater to be treated. This solution must stand for five minutes and then may be discharged into a municipal waste water treatment system.
8) *A copy of this permit, including any amendments, must be made available at all field collection sites and project sites for inspection upon request by a representative of the department or a law enforcement officer.*
9) Issuance of this permit does not absolve the permittee from compliance in full with any and all other applicable federal, state, or local laws, regulations, or ordinances.
10) A report of collecting activities, referenced to this fish resource permit number, must be submitted to the Alaska Department of Fish and Game, Division of Sport Fish HQ, P.O. Box 115525, Juneau, AK 99811-5525, Attention: Bob Piorkowski (465-6109; Robert.Piorkowski@alaska.gov), and to the AMB **(Stipulation #1)** within 30 days after the expiration of this permit. This report must summarize the number of fish captured by date, by location (provide GPS coordinates and datum), and by species, and the fate of those fish. Fish length, weight, sex, and age data should be included if collected. A completion report (abstract/background/methods /data/analysis), if not submitted with the collection report described above, must be submitted to the department within six months of the expiration of the permit. Data from such reports are considered public information. A report is required whether or not collecting activities were undertaken.

PERMIT VALIDATION requires permittee's signature agreeing to abide by permit conditions before beginning collecting activities:

_____
Signature of Permittee

cc: Audra Brase, Division of Sport Fish, Fairbanks
Cal Skaugstad, Division of Sport Fish, Fairbanks
Bonnie Borba, Division of Commercial Fisheries, Fairbanks
Mac McLean, Division of Habitat, Fairbanks
Fish and Wildlife Protection, Fairbanks

Appendix C: Alaska Department of Fish and Game permit, 2011

STATE OF ALASKA
DEPARTMENT OF FISH AND GAME
P.O. BOX 115525
JUNEAU, ALASKA 99811-5525

Permit #: **SF2011-068**
Expires: **12/31/2011**
Collections Report Due: **12/15/2011**

FISH RESOURCE PERMIT
(For Scientific/Educational Purposes)

This permit authorizes **Larissa Dehn** *(whose signature is required on page 2 for permit validation)*
person

of **SFOS University of Alaska Fairbanks** *agency or organization* **at** **Box 757220, Fairbanks, AK 99775** *address*

to conduct the following activities **from** July 1, 2011 **to** December 31, 2011 in accordance with AS 16.05.930:

Purpose: To investigate various aspects of the physiological cost of a parasitic infection in the target species.

Location: Chena/Salcha Rivers

Species Collected: King salmon

Method of Capture: Boat based electrofishing in collaboration with ADF&G brood stock take

Final Disposition: **CAUTION:** ***This authorization depends upon adequate run strength with ADF&G making that call.***
≤25 adult males and ≤25 adult females from each river may be collected and held with other ADF&G broodstock until ripe gametes and other biological samples are harvested and transported to a secure UAF lab for further investigation **(Stipulations #6 and #7)**
After gamete extraction, all salmon flesh of good quality should be donated to a local charity with the remaining carcasses treated as biological waste.

-Continued on Back-

COLLECTIONS REPORT DUE December 15, 2011. The report, using a data submission form furnished by ADF&G), shall include **ALL** species, numbers, dates, and locations of collection (datum/GPS coordinates in the decimal degrees format (dd.ddddd)) and disposition, and if applicable, sex, age, and breeding condition, and lengths and weights of fish handled. *It must also include the date/time the local biologist was contacted for final authorization to carry out collecting activities.* A completion report (abstract, background, methods, data, analysis), if not submitted with the collection report described above, must be submitted to the department by: ***June/2012***. Data from such reports are considered public information. The report shall also include other information as may be required under the permit stipulations section.

GENERAL CONDITIONS, EXCEPTIONS AND RESTRICTIONS

1. This permit must be carried by person(s) specified during approved activities who shall show it on request to persons authorized to enforce Alaska's fish and game laws. This permit is nontransferable and will be revoked or renewal denied by the Commissioner of Fish and Game if the permittee violates any of its conditions, exceptions or restrictions. No redelegation of authority may be allowed under this permit unless specifically noted.
2. No specimens taken under authority hereof may be sold or bartered. All specimens must be deposited in a public museum or a public scientific or educational institution unless otherwise stated herein. Subpermittees shall not retain possession of live animals or other specimens.
3. The permittee shall keep records of all activities conducted under authority of this permit, available for inspection at all reasonable hours upon request of any authorized state enforcement officer.
4. Permits will not be renewed until the department has received detailed reports, as specified above.
5. UNLESS SPECIFICALLY STATED HEREIN, THIS PERMIT DOES NOT AUTHORIZE the exportation of specimens or the taking of specimens in areas otherwise closed to hunting and fishing; without appropriate licenses required by state regulations; during closed seasons; or in any manner, by any means, at any time not permitted by those regulations.

Bob Piorkowski — Fish Resource Permit Coordinator, Division of Sport Fish
[signature] for Director, Division of Sport Fish
3/1/11 — Date

SF2011-068 ***continued (page 2 of 2)***

Authorized Personnel: The following persons may perform collecting activities under terms of this permit:

Shannon Atkinson, April Behr, Larissa Dehn, Nicole Farnham, Theresa Floyd, Paul MacGregor, Calvin Skaugstad, Trent Sutton, Brian Walker, Kelly Walker

Employees and volunteers under the direct supervision of, and in the presence of, one of the authorized personnel listed above may participate in collecting activities under terms of this permit.

Permit Stipulations:

1) The local Area Management Biologist (AMB), Audra Brase (459-7244; audra.brase@alaska.gov) Tanana River, must be contacted for final authorization **prior** to you engaging in any collecting activities. *The time/date of this contact must be included in your collections report (using the "data submission form" furnished by ADF&G).* This AMB has the right to specify methods for collecting, as well as limiting the collections of any species by number, time and location.
2) Each piece of unattended sampling gear must be; 1) labeled with the permittee's name, telephone number, and permit number, 2) located with GPS coordinates, and 3) accounted for/removed at the conclusion of sampling.
3) If anadromous fish species new to permitted streams and rivers are found, the permit holder will work closely with ADF&G to see that information is included in the database for the *Catalog of Waters Important for Spawning, Rearing or Migration of Anadromous Fishes.* Anadromous fish include *Oncorhynchus spp.*, Arctic char, Dolly Varden, sheefish, smelts, lamprey, whitefish, and sturgeon. Please direct questions to J. Johnson, 267-2337 or j.johnson@alaska.gov
4) **Atlantic salmon** and other **non-native invasive aquatic species** that you encounter during your sampling should be killed. In such an event please contact the nearest ADF&G office (**Stipulation # 1**) ASAP with species identification or description, capture location or location of sighting if capture is not possible, number captured, size, and sex. Preserve and turn in the whole specimen to the nearest ADF&G office.
5) This permit will fulfill the requirements of 5AAC 41.005 – 41.060 pertaining to fish transport permits (FTP's), with the condition that the transported species be destroyed when the permit expires and not be released to the wild.
6) Note: a) This permit number and its date of expiration must be displayed on all aquaria for which you provide fish; b) this permit will expire on December 31, 2011–apply for/obtain a **FRP** that is effective on January 1, 2012 if the aquaria will still contain fish.
7) **All aquaria effluent must be treated before discharge.** Fourteen ounces of household chlorine bleach must be added to and adequately mixed with each gallon of wastewater to be treated. This solution must stand for five minutes and then may be discharged into a municipal waste water treatment system.
8) *A copy of this permit, including any amendments, must be made available at all field collection sites and project sites for inspection upon request by a representative of the department or a law enforcement officer.*
9) Issuance of this permit does not absolve the permittee from compliance in full with any and all other applicable federal, state, or local laws, regulations, or ordinances.
10) A report of collecting activities, referenced to this fish resource permit number, must be submitted to the Alaska Department of Fish and Game, Division of Sport Fish HQ, P.O. Box 115525, Juneau, AK 99811-5525, Attention: Bob Piorkowski (465-6109; Robert.Piorkowski@alaska.gov), and to the AMB **(Stipulation #1)** within 30 days after the expiration of this permit. This report must summarize the number of fish captured by date, by location (provide GPS coordinates and datum), and by species, and the fate of those fish. Fish length, weight, sex, and age data should be included if collected. A completion report (abstract/background/methods /data/analysis), if not submitted with the collection report described above, must be submitted to the department within six months of the expiration of the permit. Data from such reports are considered public information. A report is required whether or not collecting activities were undertaken.

PERMIT VALIDATION requires permittee's signature agreeing to abide by permit conditions before beginning collecting activities:

_____
Signature of Permittee

cc: Audra Brase, Division of Sport Fish, Fairbanks
Cal Skaugstad, Division of Sport Fish, Fairbanks
Bonnie Borba, Division of Commercial Fisheries, Fairbanks
Mac McLean, Division of Habitat, Fairbanks
Fish and Wildlife Protection, Fairbanks

STATE OF ALASKA
DEPARTMENT OF FISH AND GAME-SPORT FISH
P.O. BOX 115525
JUNEAU, ALASKA 99811-5525

FISH RESOURCE PERMIT AMENDMENT #1
Permit No. SF2011-068

Permit Issued To: **Larissa Dehn** *(signature required below for permit validation)*

This amendment of Fish Resource Permit SF2011-068:

1) under **Authorized Personnel;** *deletes the following names:*

Trent Sutton, Brian Walker, Kelly Walker

All other conditions specified in Fish Resource Permit SF2011-068 remain in effect.

This amendment must be attached to the original permit.

Bob Clarkowski — Division of Sport Fish

May 26, 2011 — Date

PERMIT AMENDMENT VALIDATION requires permittee's signature agreeing to abide by conditions of this permit amendment:

Signature of Permittee

cc: Audra Brase, Division of Sport Fish, Fairbanks
Cal Skaugstad, Division of Sport Fish, Fairbanks
Bonnie Borba, Division of Commercial Fisheries, Fairbanks
Mac McLean, Division of Habitat, Fairbanks
Fish and Wildlife Protection, Fairbanks

STATE OF ALASKA
DEPARTMENT OF FISH AND GAME-SPORT FISH
P.O. BOX 115525
JUNEAU, ALASKA 99811-5525

FISH RESOURCE PERMIT AMENDMENT #2
Permit No. SF2011-068

Permit Issued To: **Larissa Dehn** *(signature required below for permit validation)*

This amendment of Fish Resource Permit SF2011-068:

1) *under* **Authorized Personnel:** *adds the following names:*

Kevin Fraley, Luke Mather, Collin Todd, Trevor Haynes

All other conditions specified in Fish Resource Permit SF2011-068 remain in effect.

This amendment must be attached to the original permit.

Bob Pawlowski — Division of Sport Fish

6/7/11 — Date

PERMIT AMENDMENT VALIDATION requires permittee's signature agreeing to abide by conditions of this permit amendment:

_____
Signature of Permittee

cc: Audra Brase, Division of Sport Fish, Fairbanks
Cal Skaugstad, Division of Sport Fish, Fairbanks
Bonnie Borba, Division of Commercial Fisheries, Fairbanks
Mac McLean, Division of Habitat, Fairbanks
Fish and Wildlife Protection, Fairbanks

Appendix D: Alaska Department of Fish and Game permit, 2012

STATE OF ALASKA
DEPARTMENT OF FISH AND GAME
P.O. BOX 115525
JUNEAU, ALASKA 99811-5525

Permit #: **SF2012-071**
Expires: **12/31/2012**
Collections Report Due: **12/15/2012**

FISH RESOURCE PERMIT
(For Scientific/Educational Purposes)

This permit authorizes Larissa Horstmann-Dehn *(whose signature is required on page 2 for permit validation)*
person

of SFOS/ University of Alaska Fairbanks **at** Box 757220, Fairbanks, AK 99775
agency or organization *address*

to conduct the following activities **from** July 1, 2012 **to** December 31, 2012 in accordance with AS 16.05.930:

Purpose: To investigate various aspects of the physiological cost of a parasitic infection in the target species.

Location: Chena/Salcha Rivers

Species Collected: King salmon

Method of Capture: Boat based electrofishing and 7" gill nets in collaboration with ADF&G brood stock take

Final Disposition: **CAUTION: *This authorization depends upon adequate run strength with ADF&G making that call.***

≤25 adult males and ≤25 adult females from each river may be collected and held with other ADF&G broodstock until ripe gametes and other biological samples are harvested and transported to a secure UAF lab for further investigation **(Stipulations #6 and #7)**

After gamete extraction, all salmon flesh of good quality should be donated to a local charity with the remaining carcasses treated as biological waste.

-Continued on Back-

COLLECTIONS REPORT DUE December 15, 2012. The report, using a data submission form furnished by ADF&G), shall include **ALL** species, numbers, dates, and locations of collection (datum/GPS coordinates in the decimal degrees format (dd.ddddd)) and disposition, and if applicable, sex, age, and breeding condition, and lengths and weights of fish handled. *It must also include the date/time the local biologist was contacted for final authorization to carry out collecting activities.* A completion report (abstract, background, methods, data, analysis), if not submitted with the collection report described above, must be submitted to the department by: ***June/2013***. Data from such reports are considered public information. The report shall also include other information as may be required under the permit stipulations section.

GENERAL CONDITIONS, EXCEPTIONS AND RESTRICTIONS

1. This permit must be carried by person(s) specified during approved activities who shall show it on request to persons authorized to enforce Alaska's fish and game laws. This permit is nontransferable and will be revoked or renewal denied by the Commissioner of Fish and Game if the permittee violates any of its conditions, exceptions or restrictions. No redelegation of authority may be allowed under this permit unless specifically noted.
2. No specimens taken under authority hereof may be sold or bartered. All specimens must be deposited in a public museum or a public scientific or educational institution unless otherwise stated herein. Subpermittees shall not retain possession of live animals or other specimens.
3. The permittee shall keep records of all activities conducted under authority of this permit, available for inspection at all reasonable hours upon request of any authorized state enforcement officer.
4. Permits will not be renewed until the department has received detailed reports, as specified above.
5. UNLESS SPECIFICALLY STATED HEREIN, THIS PERMIT DOES NOT AUTHORIZE the exportation of specimens or the taking of specimens in areas otherwise closed to hunting and fishing; without appropriate licenses required by state regulations; during closed seasons; or in any manner, by any means, at any time not permitted by those regulations.

Fish Resource Permit Coordinator
Division of Sport Fish

Director
Division of Sport Fish

3/9/12
Date

SF2012-071*continued (page 2 of 2)*

Authorized Personnel: The following persons may perform collecting activities under terms of this permit:

Leah Ailes, Shannon Atkinson, April Behr, Larissa Dehn, Nicole Farnham, Theresa Floyd, Kevin Fraley, Paul MacGregor, Thomas Redington, Calvin Skaugstad

Employees and volunteers under the direct supervision of, and in the presence of, one of the authorized personnel listed above may participate in collecting activities under terms of this permit.

Permit Stipulations:

1) The local Area Management Biologist (AMB), **Audra Brase** (459-7244; audra.brase@alaska.gov) Tanana River, must be contacted for final authorization **prior** to you engaging in any collecting activities. *The time/date of this contact must be included in your collections report (using the "data submission form" furnished by ADF&G).* This AMB has the right to specify methods for collecting, as well as limiting the collections of any species by number, time and location.
2) Each piece of unattended sampling gear must be; 1) labeled with the permittee's name, telephone number, and permit number, 2) located with GPS coordinates, and 3) accounted for/removed at the conclusion of sampling.
3) Gill nets must be constantly/closely monitored to minimize incidental fish, bird, and mammal mortalities.
4) Electroshocking is currently discouraged, but not prohibited. Electroshockers may not be used in anadromous waters in the presence of adult salmonids including trout or char. In areas where other means of capture are not feasible, only one pass is allowed. To obtain population estimates, sections of smaller streams not contained adult salmonids may be blocked off with seines and electroshocked. All electroshocked fish should be monitored before release with mortalities or injuries reported on the data submission form. Crew Leaders must have proof of attending formal class/field training along and ten days of electroshocking experience while crew members should have formal training.
5) If anadromous fish species new to permitted streams and rivers are found, the permit holder will work closely with ADF&G to see that information is included in the database for the *Catalog of Waters Important for Spawning, Rearing or Migration of Anadromous Fishes*. Anadromous fish include *Oncorhynchus spp.*, Arctic char, Dolly Varden, sheefish, smelts, lamprey, whitefish, and sturgeon. Please direct questions to J. Johnson, 267-2337 or j.johnson@alaska.gov
6) **Atlantic salmon** and other **non-native invasive aquatic species** that you encounter during your sampling should be killed. In such an event please contact the nearest ADF&G office (**Stipulation # 1**) ASAP with species identification or description, capture location or location of sighting if capture is not possible, number captured, size, and sex. Preserve and turn in the whole specimen to the nearest ADF&G office.
7) This permit will fulfill the requirements of 5AAC 41.005 – 41.060 pertaining to fish transport permits (FTP's), with the condition that the transported species be destroyed when the permit expires and not be released to the wild.
8) Note: a) This permit number and its date of expiration must be displayed on all aquaria for which you provide fish; b) this permit will expire on December 31, 2012–apply for/obtain a FRP that is effective on January 1, 2013 if the aquaria will still contain fish.
9) **All aquaria effluent must be treated before discharge.** Fourteen ounces of household chlorine bleach must be added to and adequately mixed with each gallon of wastewater to be treated. This solution must stand for five minutes and then may be discharged into a municipal waste water treatment system.
10) *A copy of this permit, including any amendments, must be made available at all field collection sites and project sites for inspection upon request by a representative of the department or a law enforcement officer.*
11) Issuance of this permit does not absolve the permittee from compliance in full with any and all other applicable federal, state, or local laws, regulations, or ordinances.
12) A report of collecting activities, referenced to this fish resource permit number, must be submitted to the Alaska Department of Fish and Game, Division of Sport Fish HQ, P.O. Box 115525, Juneau, AK 99811-5525, Attention: Bob Piorkowski (465-6109; Robert.Piorkowski@alaska.gov), and to the AMB **(Stipulation #1)** within 30 days after the expiration of this permit. This report must summarize the number of fish captured by date, by location (provide GPS coordinates and datum), and by species, and the fate of those fish. Fish length, weight, sex, and age data should be included if collected. A completion report (abstract/background/methods /data/analysis), if not submitted with the collection report described above, must be submitted to the department within six months of the expiration of the permit. Data from such reports are considered public information. A report is required whether or not collecting activities were undertaken.

PERMIT VALIDATION requires permittee's signature agreeing to abide by permit conditions before beginning collecting activities:

_____
Signature of Permittee

cc: Audra Brase, Division of Sport Fish, Fairbanks
Bonnie Borba, Division of Comfish, Fairbanks
Fish and Wildlife Protection, Fairbanks
Cal Skaugstad, Division of Sport Fish, Fairbanks
Will Morris, Division of Habitat, Fairbanks